Development of a technique for fish feed digestibility estimations using microtracers

by

N. M. Jahangir Kabir
B. Sc. Fisheries (Honours)
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in Aquaculture (by Research)

Department of Aquaculture
University of Tasmania
Launceston, Tasmania
Australia

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any institute, college or university, and to the best of my knowledge and belief, it contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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Abstract

The aim of this thesis was to compare the methods of feed digestibility determination in fish and subsequently develop a technique for fish feed digestibility estimations using microtracer. The review of current methods showed that none of the existing methods fulfil adequately the criterion of an ideal method when employing external markers.

Therefore microtracer F-Ni was used in this study and a series of experiments was conducted to evaluate the digestibility coefficients in fish (rainbow trout, *Oncorhynchus mykiss*) using microtracer F-Ni as a marker and compare its suitability with the conventional Cr_2O_3 and acid insoluble ash (AIA) markers.

The particle counts of microtracer F-Ni per unit weight were validated in Experiment 1, as it was essential to establish a consistent count of particles per unit weight for the calculation of digestibility values. Both direct counting and colour development methods were used for this purpose. Results showed that microtracer F-Ni marker contained on average 64 particle per mg.

The sequential uniformity in the rate of passage of the microtracer through the digestive tract were tested in Experiment 2, using similar diets with four inclusion levels to select one level for further digestibility studies. One percent inclusion level was found to be the best as the rate of increase in concentration in different parts of the digestive tract and feces were sequential and progressive for all the three days tested.

Apparent digestibility coefficients (ADC) of protein and dry matter were determined and compared employing different fecal collection procedure and different collection interval using this marker with chromic oxide

marker and acid insoluble ash marker in Experiment 3. Results indicated that the ADC values generated by using microtracer F-Ni were consistent and in close agreement with the ADC values calculated by using chromic oxide as marker in all the situations. The ADC determined by using AIA were inconsistent and higher than the ADC calculated by both chromic oxide and microtracer F-Ni markers. When microtracer F-Ni and chromic oxide were incorporated into the same diet, ADC values calculated were higher than values obtained with individual marker inclusion.

In Experiment 4, protein and dry matter digestibility of fish meal and sunflower meal ingredients were determined and compared using microtracer F-Ni, Cr_2O_3 and AIA markers. Digestibility coefficients of nutrients generated using microtracer resulted ADC values similar ($P > 0.05$) to those using chromic oxide as external marker. Experimental diets containing either microtracer F-Ni or chromic oxide supported excellent growth performances and feed conversion efficiency.

In summary, microtracer F-Ni incorporated at 1% can be used as an external marker in fish feed digestibility studies in rainbow trout.

Chapter One

General Introduction

1 Introduction

Generally the more intensive an aquaculture system, the greater the importance of supplementary feeding, and the greater the proportion of feed costs to total production costs (Hepher, 1988). According to Wee and Tacon (1992), feed and feeding cost is the single largest operating cost item in fish culture enterprise and can range from 40% to 60% of the total operating cost of production.

In order to maximise production yields at the lowest cost, cost-effective feeds capable of supporting optimum growth must be available. To formulate feeds that will meet the animals nutrient requirements, it is essential to know the nutritional requirements of the species of fish as well as knowing the nutritive value of the ingredients (Wee and Tacon, 1992). It is also important to evaluate the biological availability of the nutrients contained within each ingredient sources to the fish species cultured because although the nutrient profile of an ingredient may appear good, if these nutrients are not biologically available or not digested, they are of little value to the animal (Tacon, 1990).

The most important and simplest aspect in the evaluation of the biological effectiveness of a nutrient in feedstuffs by the fish is the determination of its digestibility (Hanley, 1987). The measurement of digestibility as a proportion of total food intake is the initial step in assessing the nutrient or energy level available to the animal for growth. In addition to being a necessary factor in nutrient and energy budgeting, digestibility in itself is an important parameter in diet evaluation and feeding ecology studies (Welch, 1968). Feedstuff digestibility assessment is essential for

(1) least-cost diet formulation, (2) nutrient requirement research, (3) screening of feedstuffs for potential nutritive value in relation to raw material quality and processing and storage conditions, and (4) formulation of diets to minimise water pollution (Hajen et al., 1993). Apart from its use in aquaculture, digestibility studies of naturally ingested food material of natural or quasi-natural populations have proved useful to evaluate and understand the success and failure of a species (Bowen, 1981; De Silva, 1985a).

Digestion is defined as the processes by which the ingested food materials are broken down into nutrients which can be absorbed across the gut wall into the vascular system (De Silva, 1989). These processes are essentially enzymatic with mechanical and acidic mechanisms also playing major roles in certain species (Wee, 1992). The biochemistry of enzyme digestion and nutrient absorption in fish have been adequately reviewed by Fange and Grove (1979), Hepher (1988) and Steffens (1989) and have been shown to be qualitatively similar to those reported for higher vertebrates. However despite the similarity in the types of enzymes they secrete, fish differ in their ability to digest feed. Some of the factors affecting the capacity of fish to digest food are the species, age, size; physiological and nutritional condition such as starvation, forced vs *ad libitum* feeding; some associated with the environmental conditions, such as water temperature, stocking density; and some are related to the food, viz., its quality and composition, particle size, amount eaten and frequency of feeding (Hastings, 1969).

Digestibility, as defined by Schneider and Flatt (1975) is the percentage of ingested nutrients which are not rejected as feces.

The digestibility of feedstuff measured as such is also called apparent digestibility and when this fraction is expressed as a percentage of the ingested amount it is known as the apparent digestibility coefficient (ADC). The term "apparent" is used because a small proportion of the fecal output is derived from the endogenous sources, eg. enzymes and mucoproteins, desquamated mucosa cells regardless of diets (Hepher, 1988). However, digestion of this endogenous protein is not complete and part of it voided in the feces. The inclusion of this metabolic protein with the dietary protein in the feces causes a bias of the digestibility coefficients toward lower values (Calow & Fletcher, 1972). In order to determine the true digestibility, the amount of metabolic fecal protein should be first determined and subtracted from the protein in the feces. Determination of metabolic fecal nitrogen in the aquatic environment is rather difficult and in doing so, some fishes do not accept non-protein diet, which makes the determination more troublesome. Considering the difficulties to measure metabolic fecal protein and its effect on digestibility, the use of "apparent digestibility coefficient" had been widely accepted in nutritional studies. Digestibility coefficients can be determined as total digestibility of feed, but since the digestibility can be different for its different nutrients, it is usually determined separately for each of the nutrients.

The digestibility coefficients can be determined directly or indirectly. Directly by determining the amount of feed (or nutrient) ingested and voided as feces.

$$ADC = \frac{\text{Food or nutrient ingested - Food or nutrient egested}}{\text{Food or nutrients ingested}} \times 100$$

The direct method of digestibility determination in fish have been used by Tunison et al. (1942), Phillips et al. (1948) and Post et al. (1965) amongst others.

Tunison et al. (1942) determined digestibility after collecting feces by filtering the water and also analysing the adjacent water using brook trout (*Salvelinus fontinalis*, Mitchell) as test animal. The method required much analysis and calculation was not very accurate. In addition errors could have arisen because of contamination of feces by N-compounds excreted in urine or across the gills (Austreng, 1978). To overcome the risk of N-contamination and nutrient losses in water, Phillips et al. (1948) force-fed trout with gelatine capsules containing known amounts of a test diet. After various intervals, fish were killed and contents of their digestive tracts were analysed. The usefulness of this method is limited by the fact that it is necessary to kill the fish and by the difficulty of estimating how far digestion has proceeded in an individual before it is killed. Smith (1971) described a direct method, adopted by Post et al.(1965) about measuring digestibility using a metabolism chamber. With this system, the collection of gill excretions and urine as well as feces are possible but the test fish suffer much stress and restriction of swimming activity and force-feeding is necessary which are the major drawbacks of this system.

This direct method demands that the food consumed and fecal material voided are accurately determined, collected and quantified. As with the fish the food is given in water and feces are egested into water, complete collection of fecal material is at best tedious and in many circumstances impossible (Atkinson et al,

1984) and also as nutrients of both food and feces may be dispersed and dissolved in water, the direct method is subjected to a high degree of experimental error (De Silva, 1989).

In order to overcome the drawbacks of the direct method, many researchers have been practicing an indirect method using inert indicators. In this method a certain amount of inert substance or marker, is used as reference compound to monitor chemical (hydrolysis and synthesis) and physical (flow) aspects of digestion (Owens and Hanson, 1992). With the use of the marker, the concentrations of the nutrient and the inert indicator are determined in both the food and the feces and the apparent digestibility coefficient (ADC) of the nutrient is calculated by monitoring the relative changes in marker concentration within the feces relative to the dietary marker concentration and the nutrient to be monitored (Maynard and Loosli, 1969).

Therefore,

$$ADC(\%) = 100 - \frac{\%\ \text{indicator in food}}{\%\ \text{indicator in feces}} \times \frac{\%\ \text{nutrient in feces}}{\%\ \text{nutrient in food}} \times 100.$$

The advantage of this method over the previous one is that there is no need to determine the amount of food ingested nor the amount of feces egested. It is sufficient to analyse a representative sample of both and determine the percentage contents of nutrient and indicator. The validity of such estimates depends on the assumption that, the inert material introduced does not interfere with the digestive metabolism of the experimental subject or its microbial population, it is not absorbed or metabolised and the rate of its passage through the gut is the same as the experimental

feed (Maynard and Loosli, 1969), it must have a specific and sensitive method of estimation (Owens and Hanson, 1992) and it should not be toxic (De Silva, 1989).

Knapka et al. (1967) described that Edin (1918) was the first to introduce chromic oxide (Cr_2O_3) as an indicator for the digestibility studies in domestic animals. Since then a wide variety of markers have been used in digestibility estimation in fish. Markers could also be of two forms; artificial foreign substances (external markers) introduced into the feed in small quantities or substances (internal markers) that are a component of the feed itself. The substances used as internal marker in fish digestibility studies are acid insoluble ash (Atkinson et al., 1984; Bowen, 1981; De Silva and Perera, 1983 and De Silva et al., 1984), hydrolysis resistant organic matter (Buddington, 1979; De Silva et al., 1984), crude fibre (Buddington, 1979; Tacon et al., 1983; De Silva et al., 1984 and De Silva and Perera, 1983) , ash (De Silva et al., 1984), cellulose (Buddington, 1979 and Van Keulen & Young, 1977), and silica (Hirao et al., 1960). The most commonly used external marker is chromic oxide (Cho et al., 1985) and less commonly used markers are polyethylene (Tacon and Rodrigues, 1984), radio isotope markers (Calow and Fletcher, 1972), metallic iron powder (Talbot and Higgins, 1983), celite (Atkinson et al., 1984), titanium IV oxide (Lied et al., 1979) and ^{32}P (insoluble ammonium molybdate) (Hirao et al., 1960).

Since the introduction of the indirect method into fish nutrition research, the problem of collecting representative unadulterated feces samples has not yet been solved. A critical review of methods for feces collection has been given by Satoh et al. (1992). Windell et

al. (1978) removed feces, which had settled in the tank, simply by using a hand net in intervals of 1 - 4 h . In the 'Guelph system' (Cho and Slinger, 1979) the effluent of the experimental tank passes through a settling column, from which feces are easily and quantitatively removed. French authors have tried to construct automatic continuous feces samplers with the purpose of minimising the contact of released feces with waters (Choubert et al., 1982). Smith (1971) developed a metabolism chamber for fish which permitted separate and quantitative collection of feces, urine and gill excretions. Spyridakis et al. (1989) used immediate pipetting in tank water, continuous filtration and decantation for collecting feces and they recommend continuous filtration as most appropriate technique for digestibility trials with sea bass.

However, leaching of nutrients from the fecal material into the water seems to be inevitable with all of the methods mentioned above, resulting in an over estimation of the digestion coefficients measured (Vens-Cappell, 1985). This is the major reason why numerous authors have tried to avoid the contact of feces with water by developing sophisticated methods. These methods of feces collection include : dissection (Austreng, 1978; Windell et al., 1978; Henken et al, 1985; and Spyridakis et al., 1989), stripping (Austreng, 1978; Windell et al., 1978; Spyridakis et al., 1989; and Vens-Cappell, 1985) and suction (Lovell, 1977; and Spyridakis et al., 1989). Objections to all these methods of direct feces sampling from intestine relate to the fact that fecal material may be removed prior to the completion of the natural retention time, thereby reducing the digestion and absorption capacity and simulating poor digestibility (Vens-Cappell, 1985). Contrary to this concept, Austreng (1978)

obtained higher digestion coefficients for all nutrients when samples were stripped only from the hindmost part of the rectum. These coefficient values were even higher than those sampled by extracting the feces by dissection of the same part. Windell et al.(1978) commented that errors might be introduced in Austreng's (1978) work due to the contamination by sperm, ova or mucus. The frequent handling of fish for stripping may also cause stress which is sometimes expressed as diarrhoea and incomplete food digestion (Hepher, 1988). Dissection also indicate the need to sacrifice the fish in each experiment. Hence it follows that there is presently no method for feces collection without any inherent errors.

Another most disturbing sources of error of the inert indicator method are, however, related to the indicator used. To be an effective indicator, a substance must be indigestible and unabsorbable, remain homogenously mixed with the digesta during passage through the gut, and have no effect on the digestive metabolism of the animal (Schneider and Flatt, 1975). No single marker fulfils all these criteria, but the tolerable degree of error differs with the variables being measured. If the feces are to be sampled from water, after being egested, the analysis is subject to the same errors as the direct method due to dispersion and solution of nutrients in water. Even though Cr_2O_3 is widely used and continues to be used as an external marker, Bowen (1978) found that in *Tilapia mossambica* Cr_2O_3 moved at a different rate than the rest of the food. Haenlein et al. (1966) has also indicated a considerable diurnal variation in the excretion pattern of Cr_2O_3 in pony. Hilton et al. (1981) found that extruded pellet feed moved slower than stream pelleted feed due to the lower density of the

later. De Silva and Owoyemi (1983) reported that the specific gravity of the diet affect its rate of passage through the gut. The radioisotope technique is highly desirable from the standpoint of accuracy and time required for analytical procedure, however, the problem of radio-contamination prohibit its use in many instances. These observations raise some doubt regarding the use of external markers. Internal markers or inert components of the test diets have been employed as markers initially in ruminants (Kane, 1953; as cited by Maynard and Loosli, 1969) and in zooplankton (Conover, 1966) The advantage of this method is that it can be applied to analyses of food digestibility by fish in their natural habitats. It also eliminates handling effects which seriously modify the behaviour and physiology of the fish (Moriarty, 1973). But this method also have some drawbacks.

Buddington (1980) recommended the use of hydrolysis resistant organic matter (HROM), primarily composed of cellulose and chitin, as an internal marker for digestibility studies. However several species of fish have been reported to possesses either cellulase (Stickney, 1979) or chitinase (Danulat and Kausch, 1984), and although these may not affect HROM in specific situations (Buddington, 1980), the general assumption that this fraction of the diet is indigestible is questionable (Atkinson et al., 1984). Bowen (1981) found that a small fraction of HROM is assimilated from the detrital aggregate by *Tilapia mossambica.*

The use of crude fibre as an indicator (De Silva and Perera, 1983) can be criticised on similar grounds. Van Dyke and Sutton (1977) reported that crude fibre has shown to be assimilated in a very small extent at least by certain species.

On the other hand, the insoluble mineral component of fish diets, whether determined as silica (Hickling, 1966), hydrolysis resistant ash (Bowen, 1981) or acid insoluble ash (AIA) shows potential as an indicator (Atkinson et al., 1984). Research in ruminants (Van Keulen and Young, 1977), pig (McCarthy et al., 1974) and poultry (Vogtmann et al., 1975) has validated the use of AIA as a naturally occurring marker for digestibility trials. McCarthy et al. (1974) found that AIA digestion coefficients showed no indication of diurnal variation in pig. The same was found true for chicken and sheep also (Thonney, 1981). Thus compared to other internal indicators, AIA may be a better choice (Thonney, 1981).

The question of whether or not some indigenous reference markers are digested and absorbed has not been settled and conclusions reached by different workers contradict each other. For example, De Silva et al.(1984) are of the opinion that assimilation efficiency values obtained using ash and hydrolytic resistant organic matter are not significantly different; Bowen (1981) found that both hydrolytic resistant organic matter and ash are assimilated and digested. Chitinolytic enzyme of possibly non-bacterial origin have been observed in the digestive system of various fish species (Fange and Grove, 1979). Smith and Lovell (1973) reported 0-1.47% cellulose digestion by Channel catfish, *Ictalurus punctatus* , fed a high protein diet.

Such assimilation of marker results in underestimation of assimilation efficiency. The problem is even further complicated because of environmental variation in the composition of the diet (Getachew, 1988).

Inspite of constraints and drawbacks of the inert indicator method, most researchers are now in agreement to use this method for measuring digestibility. But there is still a controversy as to whether internal or external indicators are more suitable and or reliable.

As the conventional analytical techniques require the services of specialist laboratories, the time lag between submission of samples and receipt of results usually prevents quick action; the need to find an alternative marker for digestibility studies in fish is pressing.

Considering all the inadequacies of individual markers, this study was aimed to evaluate the use of microtracer F-Ni for digestibility estimation in fish. Microtracer F-Ni is a dye impregnated marker particle of iron and nickel, developed by Micro Tracers Inc. of San Francisco, California, USA. This tracer is easily identifiable and harmless (Shane, 1982), used in animal feed and serves as external markers for food to allow confirmation of mixing adequacy as well as to monitor proper addition of other ingredients. Ninety five percent of the particles of this tracer passes through 50 mesh (297 μm sieve) but retained on 100 mesh (149 μm sieve). It has been recommended that approximately 5 grams of the preparation is added to a ton of feed at a cost of 5 cents per ton to evaluate the mixing efficiency in the factory. The procedure of assaying food and feces containing microtracer F-Ni as a marker for digestibility studies is considerably more simple, as the tracer is separated magnetically and does not require an extraction and sedimentation technique. The principal value of the test is that they can be performed with an acceptable degree of

accuracy by relatively unskilled personnel, under field and office condition.

Therefore, the aim of this study was to evaluate the suitability of microtracer F-Ni as an external marker for digestibility studies in rainbow trout (*Oncorhynchus mykiss*) and to compare the effectiveness with another widely used external marker, Cr_2O_3 and an internal marker, acid insoluble ash.

Chapter Two

General Methodology

2.1 Microtracer measurement technique :

Microtracer F-Ni for this study was supplied by Micro Tracers, Inc., 1370 Van Dyke Avenue, San Francisco, California 94124, USA. In principle, the microtracer F-Ni is a dye impregnated ferro-nickel alloy powder with magnetic properties, passing through 50 mesh or 297 μm sieve and be caught on 100 mesh or 149 μm sieve, resisting dissolution in the gastro-intestinal tract and is recovered from a feces water slurry by a magnetic wand. The retrieved tracer is transferred from the wand to a sheet of filter paper and smeared over the paper. The filter paper were then treated with acid and colour reagents and the developed red colours, which represents individual microtracer F-Ni particles, were counted.

2.1.1 Wand :

This is a 45 cm long with a 1.27 cm diameter copper pipe, capped at both ends, having a flange 10 cm from the bottom end (the wand held vertically) and containing a free moving magnet inside (Figure 2.1.1). With the wand upright, the magnet seats between the bottom of the wand and its flange. With the wand tilted to an angle at which the magnet slid to the far end, the magnet in sliding transported ferromagnetic particulate retrieved by the wand from the area below the flange to the flange. In so doing the particulates dropped freely from the wand at the flange. Other particulates held at the very bottom of the end cap would drop from the end as the magnet sledded away.

2.1.2 Retrieval procedure :

The retrieval procedure for the microtracer F-Ni particles were performed as suggested by Anon. (1992).

a) Materials :

i) Plastic pail.

ii) Magnetic wand.

iii) Whatman #41 Filter paper, 15.0 cm.

b) Methods:

i) The dried feed and feces samples were weighed and transferred to a plastic container and a small amount of water is added to break up the sample and mixed to smooth consistency. It was further diluted to make a mobile liquid .A glass rod was used for stirring and it was thoroughly rinsed inside the pail. The upright wand was then used for final stirring for 2 - 3 minutes.

ii) With wand placed horizontal, it was washed gently with running water to remove adhering debris, then rinsed with 100% alcohol to remove the water and then air-dried.

iii) When dried, the bottom end of the wand was held over a sheet of filter paper and the wand was slowly inverted while its end and flange remained over the paper. Retrieved particulates dropped to

the paper. Once the magnet has moved to the other end of the wand, it was possible to tapped the particulates which were adhered to the wand to the filter paper.

iv) By cupping the paper, the retrieved particulates were centred on the paper, then smeared by rubbing gently with a small section of a second piece of filter paper.

The paper was then treated for development of the colour spots to be counted.

2.1.3 Colour development procedure :

The colour development procedure for the microtracer F-Ni particles were followed as suggested by Anon. (1992).

a) Materials :

i) Two 15 cm square of glass to support the filter paper.

ii) The Acid Reagent.
25 gms of tartaric acid were dissolved in distilled water to make 100 ml., then mixed with 100 ml of hydrochloric acid (3.5 N).

iii) The Colour Reagent.
1% alcoholic solution (ethyl alcohol) of dimethylglyoxine were mixed with an equal volume of ammonium hydroxide.

iv) A hot plate (griddle).

b) Methods :

i) 3 ml of acid reagent were poured on the glass plate.

ii) Holding the paper (containing the recovered microtracer particles) horizontally, it was brought into contact with the acid reagent so that the paper was completely wetted, but not in excess.

iii) After two minutes, the paper was removed from the glass plate and placed on another sheet of paper (or paper towel) to absorb as much acid reagent as possible, then it was dried on the griddle.

iv) 3 ml of colour reagent was poured on the other glass plate. Holding the paper horizontally, it was brought into contact with the colour reagent so that the paper was completely wetted. Red spots developed immediately. The spots were relatively large and diffuse and each spot indicated the presence of either iron or nickel.

v) The paper was dried as before. The diffused spots either disappeared or reduced to sharp stable red specks of nickel dimethylglyoxine.

The corresponding ferrous compound was fugitive under the specified conditions. Iron spots then would either disappeared or turned brown.

The red specks were counted. If numbers were large, ruling grid lines were used for counting. The developed colours for microtracer F-Ni particles are shown in Figure 2.1.2.

All this methods were performed under a fume cup-board.

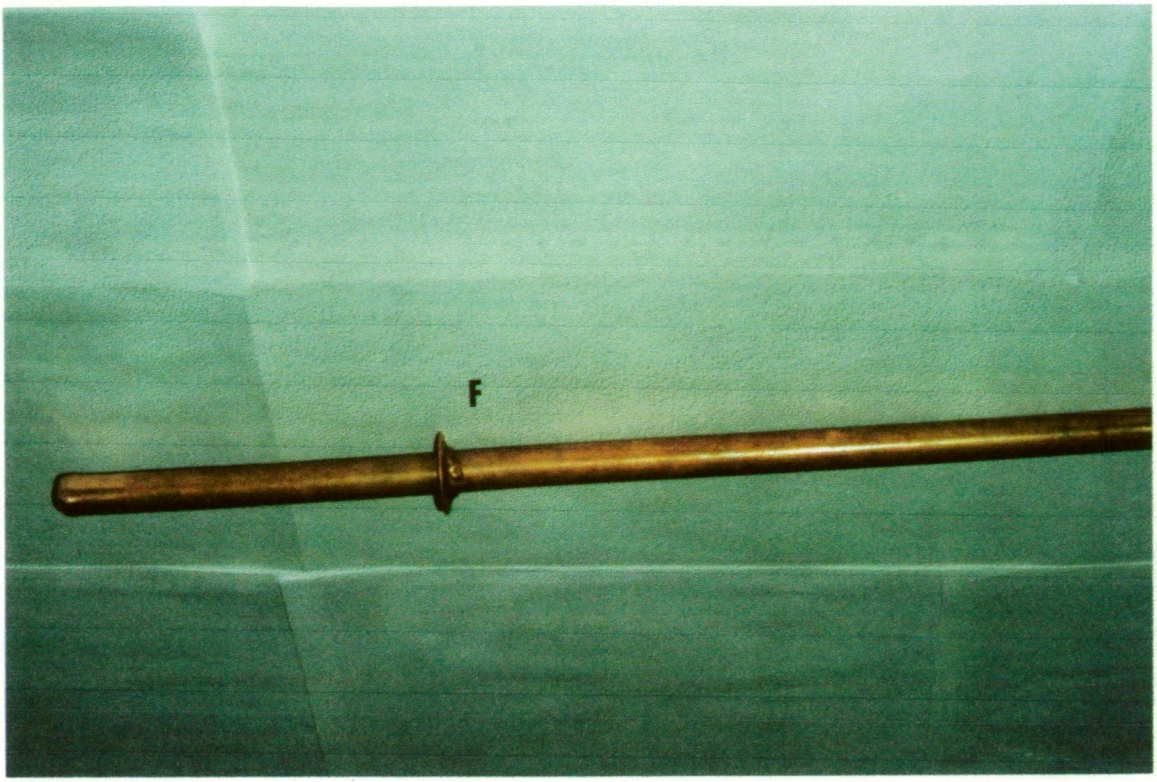

Figure 2.1.1 : The magnetic wand used for microtracer F-Ni particle recovery. F denotes flange of the wand.

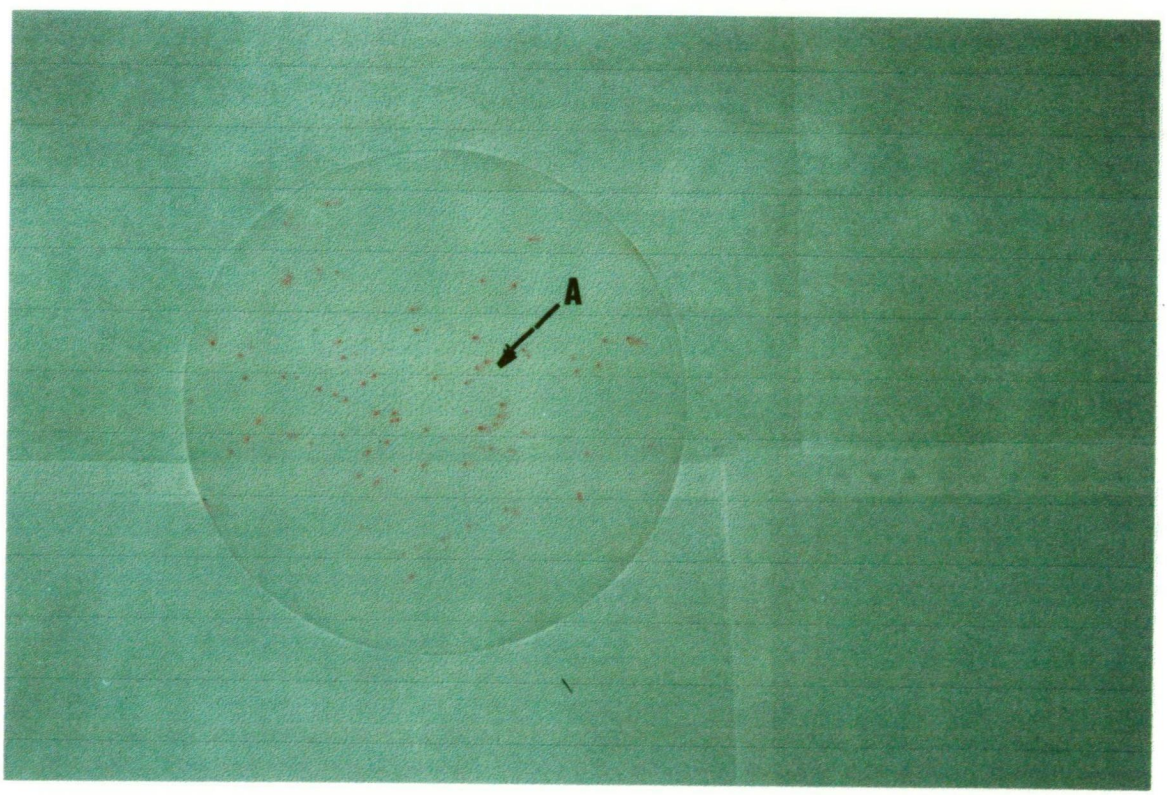

Figure 2.1.2 : The red colours from microtracer F-Ni particles (A) after development.

2.2 Experimental System

All experiments were conducted in 12 cylindro-conical fibre glass tanks (Figure 2.2.1). The tanks were located indoors at the National Key Centre for Teaching and Research in Aquaculture, University of Tasmania at Launceston Campus. Dimensions of the tank were 0.78 m in its largest diameter and 0.96 m in height with 0.90 m depth of water. Total volume of water in each tank was 0.29 m^3. Each tank was fitted with a 25 mm PVC pipe or rigid plastic tube at its conical end. A flexible plastic tube (25 mm inner diameter) was fixed on to the conical end and secured with stainless steel hose clamp to act as a feces collector. Each tank was supported by 3 wood or fibreglass pillars which allowed space to place the fecal collector tube. Circular and up welling water flow was maintained in the tank by placing the inlet (a 25 mm PVC pipe) 40 cm under water surface with the discharge of the water at an angle to the wall. The point of water intake at the outlet (a 40 mm PVC pipe) was placed in the centre of the tank. The water flow pattern allowed heavy particles, including feces to be retained in tanks and collected in the feces collectors.

The experimental tanks were housed within a recirculating system. Each tank was continuously supplied with recycled water at a mean rate of 11 l / min, sourced from a reservoir after passing through a biofilter tank. Water was aerated in the reservoir and in each tank. The biofilter tank (1.0 x 0.6 x 0.6 m^3) consisted of three layers; dacron fibre, oyster shells and followed on the bottom by cuttings of polyethylene pipe for filtering, buffering and as substrates for

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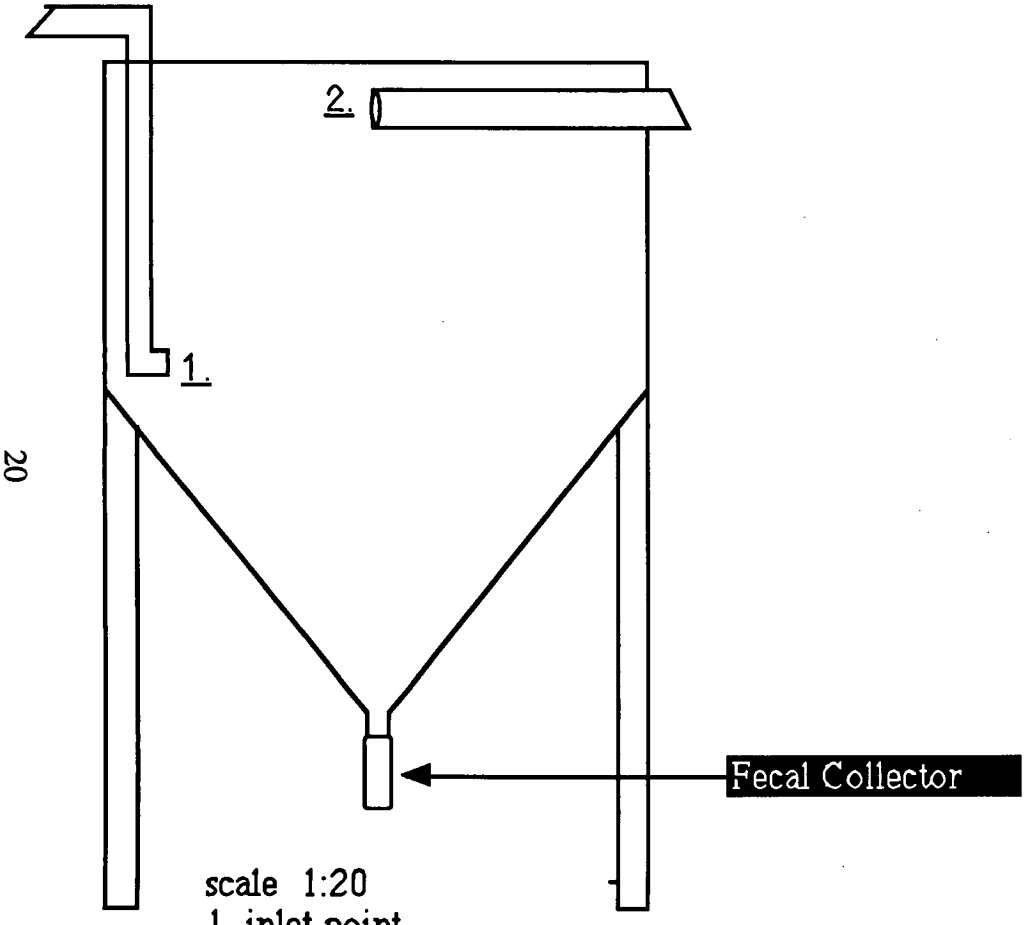
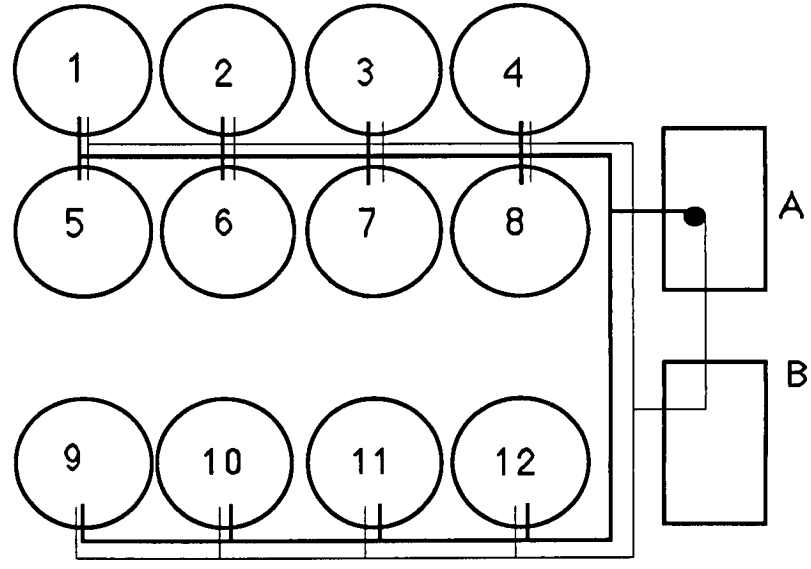


scale 1:20
1. inlet point
2. outlet point



1 - 12 experimental tanks.
A reservoir tank.
B biofiltration tank.
water flow into tanks.
water flow out from tanks.
not to scale

Figure 2.2.1. Experimental tank and system.

nitrifying bacteria. Water from the tanks was dispersed on the top of the biofiltration tanks by a piece of holed corrugated fibreglass roofing.

After the second week, oyster shells were placed in three polyethylene net-bags and sandwiched between two layers of dacron fibre to avoid pieces of shells entering the experimental tanks.

Water temperature and total ammonia level were monitored. When exceptionally high ammonia levels were recorded, up to 100% water exchange per day was effected. Normally the water exchange rate per day was up to 30%.

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the National Key Centre rearing stocks, and acclimatised in the tanks prior to the commencement of each experiment. The duration of acclimatisation before each experiment will be described in each experiment separately. To prevent fish from jumping out, each tank was covered with nylon net (1.5 - 2.0 cm in diameter).

Chapter Three

Experiment One

Validation of Microtracer F-Ni particle measurement technique

3.1.1 Introduction

Microtracer F-Ni is a ferro-nickel-magnetic alloy powder, resisting dissolution in the gastro-intestinal tract. When included with diet, the feed and feces can be assayed for microtracer qualitatively and quantitatively and can then be useful as an excellent indicator for digestibility estimations.

According to suppliers specification, the particles of microtracer F-Ni are variable in size and they passed through the 50 mesh (297 μm sieve) and be caught on 100 mesh (149 μm sieve). Considering the different particle size, it is extremely necessary to verify whether a consistent product count can be maintained or not.

The microtracer F-Ni can be included into feed on weight / weight (w/w) basis like other external markers. It is however not possible to accurately recover this tracer from the feed or feces by w/w basis directly because of possible contamination from other compounds adhering to the marker. Instead, the recovery can be done through a direct counts of developed colours of particles, i.e., the number of particles. Therefore it is necessary to establish a relationship between count of particles and weight of particles so that recovery by w/w basis can also be calculated.

Therefore, the aim of this experiment was to validate and standardise the particle counts per unit weight of microtracer F-Ni for further calculation of digestibility coefficient values.

3.1.2 Materials and methods

A known amount of microtracer F-Ni particles were weighed in a analytical balance (to two decimal points) and then spread onto a square marked paper. The particles were then counted directly by using a magnifying glass (10 X).

The particles were then transferred to a filter paper and treated with acid and colour reagents as described in chapter two. The paper was then dried over the hot plate and the developed colour spots were then counted.

3.1.3 Results

The results of the particle count are presented in table 3.1.1. Same counts were found in direct and after development procedure.

Table 3.1.1 : Counts of microtracer F-Ni particles.

Wt.of particles (mg)	Total number of particle counted.	Particles /mg
0.60	46	76.67
0.90	52	56.67
1.00	52	52.00
1.50	110	73.33
1.80	115	63.89
1.82	112	61.54
2.00	113	56.50
2.20	134	60.91
4.00	296	74.00
	Mean	63.95
	SE	2.93
	CV (%)	13.7

3.1.4 Discussion

From this study, it have been concluded that microtracer F-Ni (Lot No H-92-56774, Microtracers Inc., USA) had a consistent average particle count per unit weight, which was found to be 63.95 particles per milligram of microtracer F-Ni. For convenience 64 particles per mg were thus used as a standard for the subsequent experiments to calculate the different digestibility coefficient values as the same lot of microtracer F-Ni were used throughout the experiments.

Chapter Three

Experiment Two

Movement Pattern of Microtracer F-Ni through the digestive tract

3.2.1 Introduction

Utilisation of external markers have played a key role in advancing the understanding of digestion (Owens and Hanson, 1992). In this indirect method a certain amount of inert substances which is not digested by the fish, is introduced into the diet. Throughout the last five or six decades, substances such as polyethylene (Tacon and Rodrigues, 1984), metallic iron powder (Talbot and Higgins, 1983), celite - a form of diatomaceous silica used to supplement HRA (Atkinson et al., 1984) and some other substances have been used as external marker in digestibility studies with varying degrees of success. The most widely used external marker in assimilation studies of fish and other animals is chromic oxide (Cho et al., 1985).

Calow and Fletcher (1972) presented a series of stipulations that must be met in order to establish a marker as ideal one. One of this stipulations is that a marker must remain homogeneously mixed with the digesta during passage through the gut. In other words, an ideal marker should not move along the gut of the animal at a differential velocity from the rest of the food material (De Silva, 1989). This conditions are not always met by the presently used external markers.

A variation have been reported on the differential movements of Cr_2O_3 although it is the most commonly used external marker at this moment. Knapka et al. (1967) showed that in a mammal (donkey) the excretion of chromic oxide is not always complete and can vary during the day. Digestibility coefficients based on Cr_2O_3 were significantly lower than those based on other indicators. Diurnal variation in the excretion of chromic oxide has also been reported in

pony (Haenlein et al., 1966). Also, in fish it was found that chromic oxide does not always move through the digestive tract at the same rate as the food (Bowen, 1978).

Henken et al. (1985) determined digestibility of dry matter, crude protein and gross energy by African catfish (*Clarias gariepinus*) using the direct method and chromic oxide indicator method under identical conditions. The latter method gave lower values although the chance of leaching of nutrients was equal for both. Tacon and Rodrigues (1984), who compared apparent digestibility coefficients of rainbow trout using chromic oxide at three dietary concentrations (0.5, 1.0 and 2%), found that with 2% inclusion level nutrient digestibility coefficients were significantly higher than those for fish fed the lower chromic oxide levels. They explained this by proposing that chromic oxide at the higher inclusion level passed through the gastro-intestinal tract at a faster rate relative to the digesta.

Compounds disappear from the digestive tract by digestion or passage. Thus rate of disappearance from a given site depends on the food particles rate of digestion and rate of passage. Indigestible compounds disappear only because of passage. As the nutrients in feeds were digested and absorbed, the corresponding concentration of indigestible fractions, such as markers, increases in the digesta. The disappearance of food and increase of markers resulted in an increase of digestibility values proportionately. Austreng (1978) found that the protein digestibility in stomach was the lowest whereas in the distal end of rectum it was the highest but in his result he didn't mentioned about the marker concentration strategy. However, it is a general assumption that the marker concentration in

the digesta at the distal end should increase proportionately according to decrease of the amount food by absorption and this increase should follow a sequential uniform pattern.

This experiment was designed to test whether the movement of microtracer F-Ni along the different parts of the digestive tract follows a sequential uniform pattern in relation to the concentration of food and digesta at 0.25, 0.50, 1 and 2 percent inclusion levels. Another objective was to select a particular inclusion level of this tracer for further digestibility studies.

3.2.2 Materials and Methods

3.2.2.1 Diets

Four experimental diets were made by grinding the commercial trout pellet (manufactured by Gibson Feed Mill, Tasmania, Australia) in a coffee grinder into a powder form and mixed with microtracer F-Ni at 0.25, 0.50, 1.00 and 2.00 percent levels respectively on air-dry basis for testing the efficiency of mixing and movement of tracer along the gut. Prototek™ (a seaweed extract, manufactured by Algae Produkter a/s, PO Box 494, N 3002, Drammen, Norway) was also incorporated into the mixture to serve as a binder at 1% level. The dry ingredients were mixed in a Atlas Food Mixer (model V-20) and blended for 20 minutes. The process was repeated with the addition of water until the binder had been primed. The homogeneous paste was then extruded under pressure through a 3.18 mm die plate in a Kenwood Major Mincer (model KM 230), forming long spaghetti-like strands. These were then dried by warm air currents in a drying chamber (40°C) for 24 hours and

subsequently broken into pellets approximately 0.5 - 1.0 cm in size. The dry pellets were then sealed in polyethene bags and stored in a cool room at temperature around 0°C. A total of 3 kilograms of each experimental diet were prepared. Three replicates for each diet were allocated.

3.2.2.2 Fish and the experimental system

The average weight of the test fish, rainbow trout (*O. mykiss*) used was 23.21 g. (standard deviation 4.09 g.) and they were distributed randomly between the tanks at a stocking density of 8 fish per tank. The experiment was conducted by using all the 12 cylindroconical tanks, as described in Chapter 2, arranged in triplicate random treatments. Before commencement of experiment, fishes were acclimatised to the rearing system for one week and during that time they were fed with commercial trout pellet (Gibson Feed Mill, Tasmania).

3.2.2.3 Feeding and fecal collection

Fish were starved initially for 24 h prior to the beginning of the experiment. They were then fed every 48 h. After each feeding time (0, 48 and 96 h after the end of initial starvation period) two fish from each tank were sampled, without replacement, 6 h later (6, 54 and 102 h after the end of initial starvation period). There were three tanks for each of the four diets and a total of six of the eight fish in each tank were used. From the sampled fish, contents of stomach, intestine and rectum were collected and kept separately. At the time of sampling fish, feces from respective fecal collectors were also collected. The collected samples were dried in the oven at 80°C

for 24 h, weighed in a analytical balance and stored in a air-tight cabinet.

3.2.2.4 Analytical procedure

The microtracer F-Ni particles from all the collected samples were recovered by the magnetic wand and subsequently developed by acid and colour reagents, as described in chapter 2, for counting. Particles per milligram of digesta or fecal sample (on moisture free basis) were calculated and the amount of microtracer particles (% w/w) were calculated by dividing particles / mg by 64 (1 mg of micotracer = 64 particles) and then multiplied by 100.

3.2.3 Results

The results of microtracer F-Ni recovery from different parts of the digestive tract and faeces in different days are presented in Table 3.2.1 and Figures 3.2.1 - 3.2.4.

Table 3.2.1 : Microtracer F-Ni marker concentration (% w/w) as recovered from different parts of digestive tract of rainbow trout fed diets containing varying amounts of microtracer.

Inclu -sion level	Meas -ured level	Day	Stomach[1]	Intestine[1]	Rectum[1]	Feces[2]
0.25	0.22	1	0.23 (0.06)	0.31 (0.06)	0.65 (0.12)	0.39 (0.11)
0.25	0.22	2	0.27 (0.05)	0.43 (0.07)	0.37 (0.09)	0.43 (0.04)
0.25	0.22	3	0.49 (0.13)	1.27 (0.32)	1.24 (0.41)	0.38 (0.03)
0.50	0.46	1	0.54 (0.06)	0.82 (0.18)	1.49 (0.35)	1.68 (0.26)
0.50	0.46	2	0.42 (0.05)	0.55 (0.23)	1.64 (0.62)	0.70 (0.05)
0.50	0.46	3	0.75 (0.07)	0.89 (0.15)	3.49 (0.81)	1.51 (0.14)
1.00	0.88	1	1.13 (0.11)	1.73 (0.39)	2.38 (0.53)	2.64 (0.34)
1.00	0.88	2	0.82 (0.15)	1.25 (0.18)	1.59 (0.32)	1.82 (0.22)
1.00	0.88	3	1.90 (0.56)	2.61 (0.40)	3.66 (0.71)	4.04 (0.15)
2.00	1.87	1	1.32 (0.41)	1.98 (0.54)	2.09 (0.19)	4.46 (0.19)
2.00	1.87	2	1.14 (0.33)	1.29 (0.51)	1.62 (0.27)	3.73 (1.32)
2.00	1.87	3	1.97 (0.30)	3.32 (0.67)	5.61 (1.38)	6.41 (1.04)

[1]Values represent mean with s.e.m. in the parentheses, n=6.
[2]Values represent mean with s.e.m. in the parentheses, n=3.

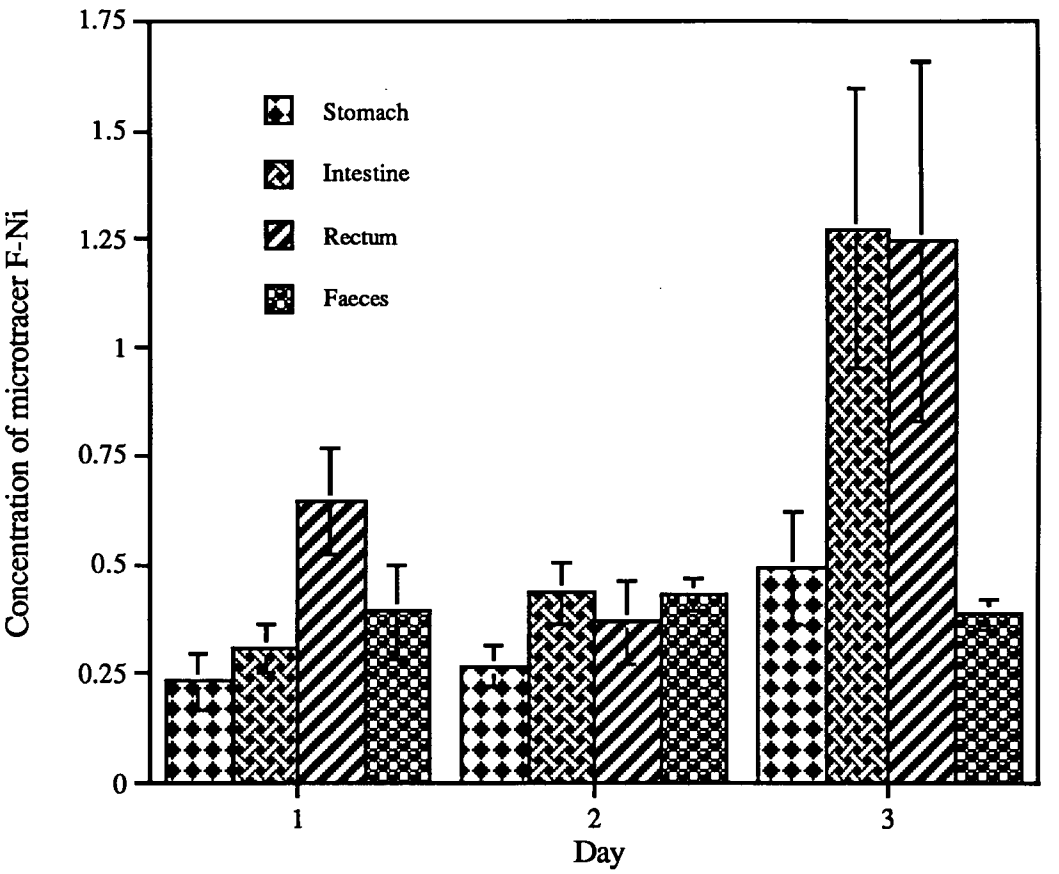


Figure 3.2.1 : Concentration of microtracer F-Ni (mean ± s.e.m., n=3-6, expressed as % w/w basis) recovered from different parts of the digestive tract of rainbow trout fed experimental diets containing 0.25% microtracer F-Ni.

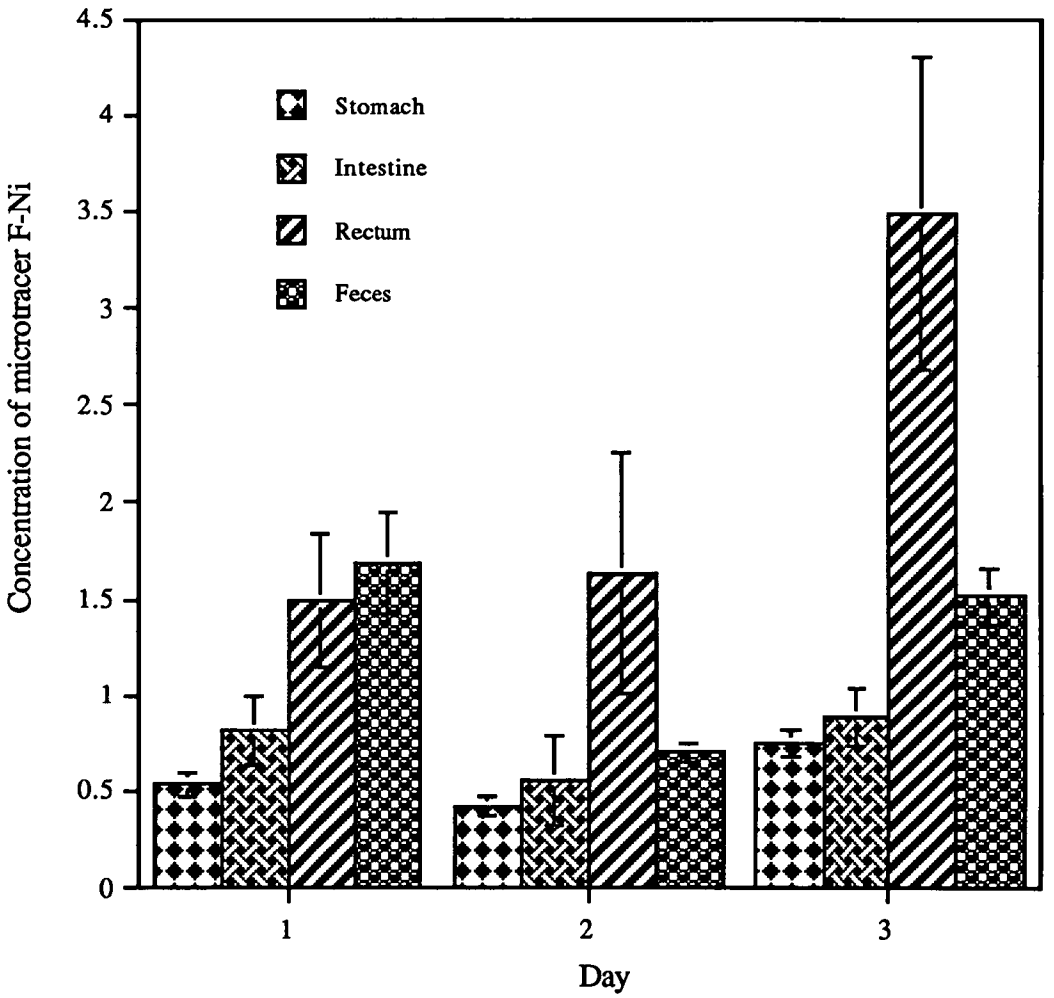


Figure 3.2.2 : Concentration of microtracer F-Ni (mean ± s.e.m., n=3-6, expressed as % w/w basis) recovered from different parts of the digestive tract of rainbow trout fed experimental diets containing 0.50% microtracer.

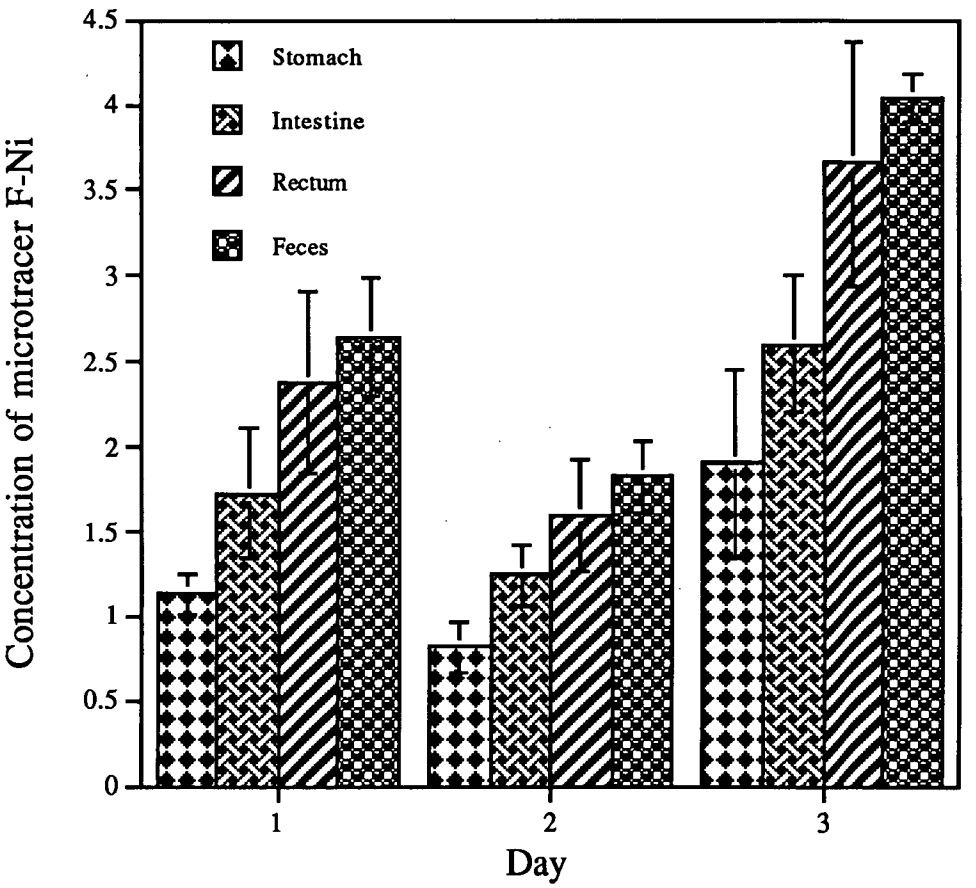


Figure 3.2.3 : Concentration of microtracer F-Ni (mean ± s.e.m., n=3-6, expressed as % w/w basis) recovered from different parts of the digestive tract of rainbow trout fed experimental diets containing 1.00% microtracer.

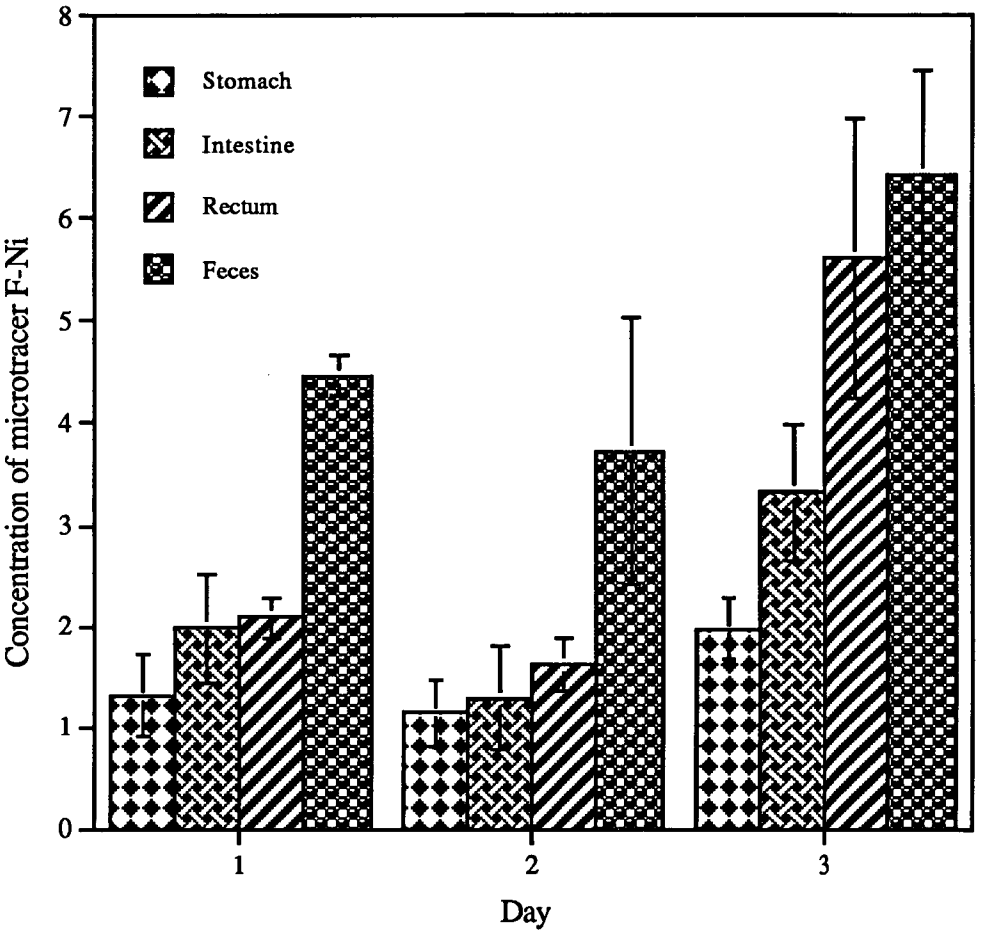


Figure 3.2.4 : Concentration of microtracer F-Ni (mean ± s.e.m., n=3-6, expressed as % w/w basis) recovered from different parts of the digestive tract of rainbow trout fed experimental diets containing 2.00% microtracer.

The percentage increase of microtracer F-Ni concentration in different parts of the digestive tract as compared with the concentration in diets are shown in Table 3.2.2 and Figures 3.2.5 to 3.2.8.

Table 3.2.2 : Percentage increase of microtracer F-Ni concentration in stomach, intestine, rectum and feces as compared with the concentration of this tracer in diets.

Inclu -sion level	Meas -ured level	Day	Stomach	Intestine	Rectum	Feces
0.25	0.22	1	5.23	40.95	193.27	78.77
0.25	0.22	2	21.18	97.18	67.09	96.18
0.25	0.22	3	123.23	478.59	465.18	74.82
0.50	0.46	1	16.41	77.26	224.78	266.17
0.50	0.46	2	- 8.89	20.52	255.52	52.04
0.50	0.46	3	63.20	92.57	658.59	229.24
1.00	0.88	1	28.78	96.24	170.14	200.30
1.00	0.88	2	- 6.49	41.5	80.92	107.30
1.00	0.88	3	116.26	196.05	315.58	359.10
2.00	1.87	1	- 29.40	5.99	11.61	138.61
2.00	1.87	2	- 39.01	- 30.83	- 13.28	99.40
2.00	1.87	3	5.50	77.54	200.06	242.93

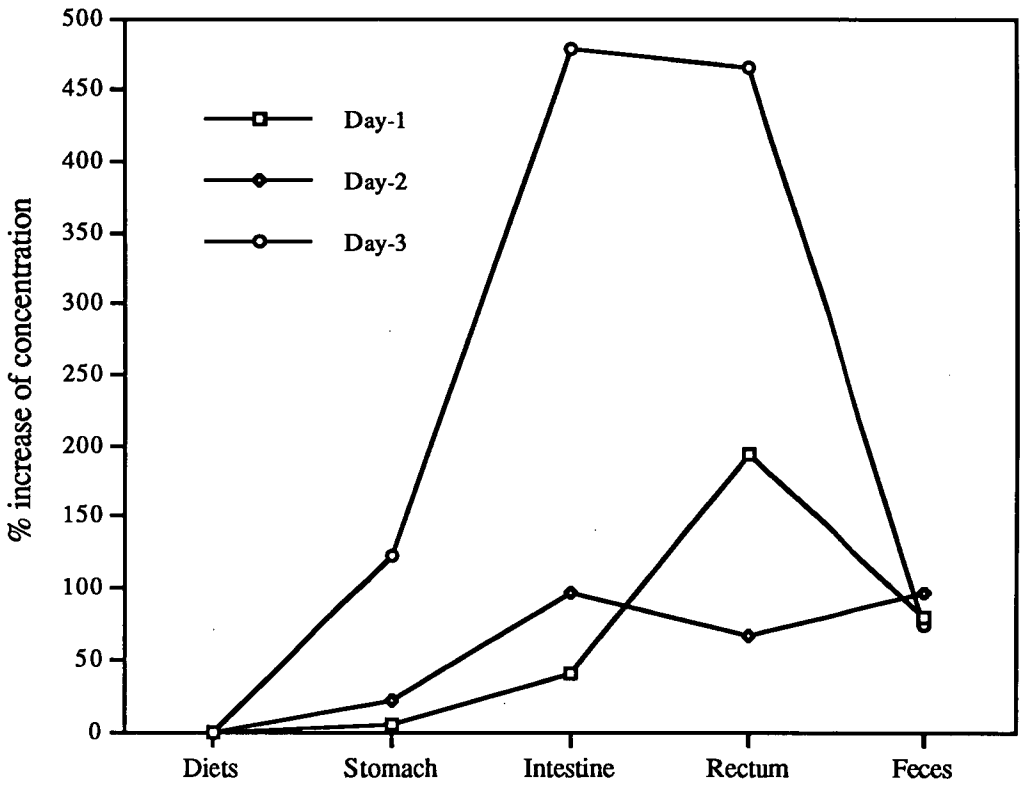


Figure 3.2.5 : Percentage increase of microtracer F-Ni at different parts of the digestive tract of rainbow trout fed experimental diet with 0.25% (w/w) microtracer F-Ni, as compared with the concentration of this marker in diet.

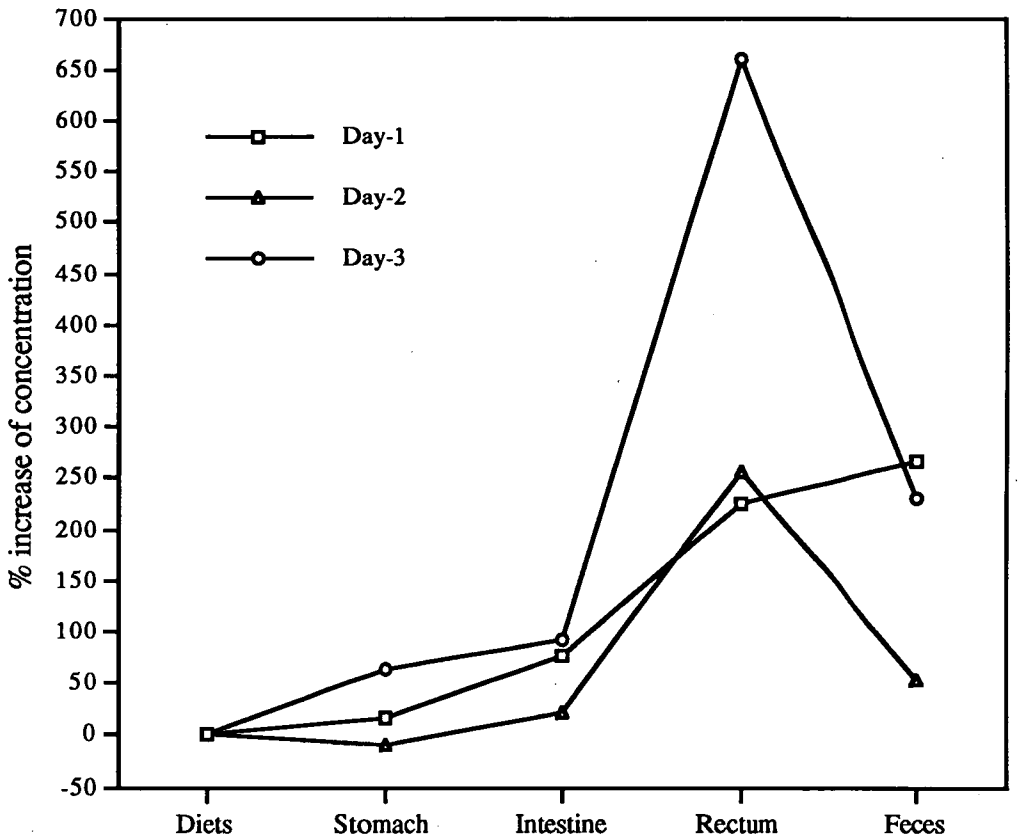


Figure 3.2.6 : Percentage increase of microtracer F-Ni at different parts of the digestive tract of rainbow trout fed experimental diet with 0.50% (w/w) microtracer F-Ni, as compared with the concentration of this marker in diet.

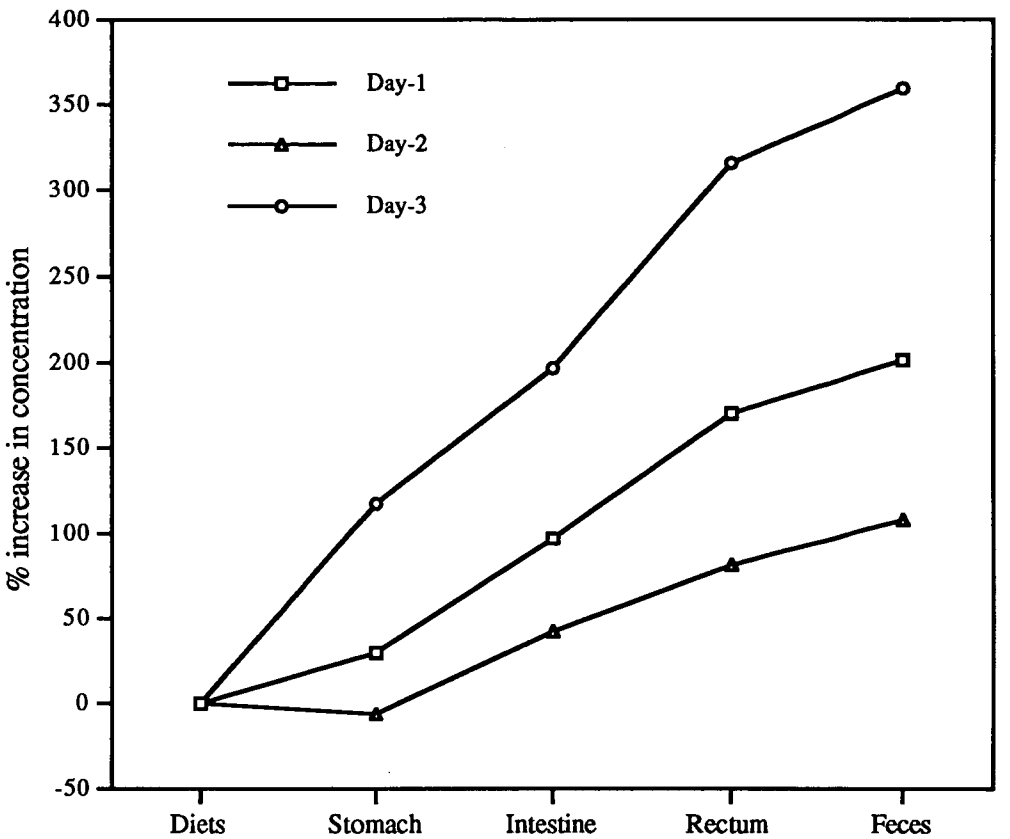


Figure 3.2.7 : Percentage increase of microtracer F-Ni at different parts of the digestive tract of rainbow trout fed experimental diet with 1.00% (w/w) microtracer F-Ni, as compared with the concentration of this marker in diet.

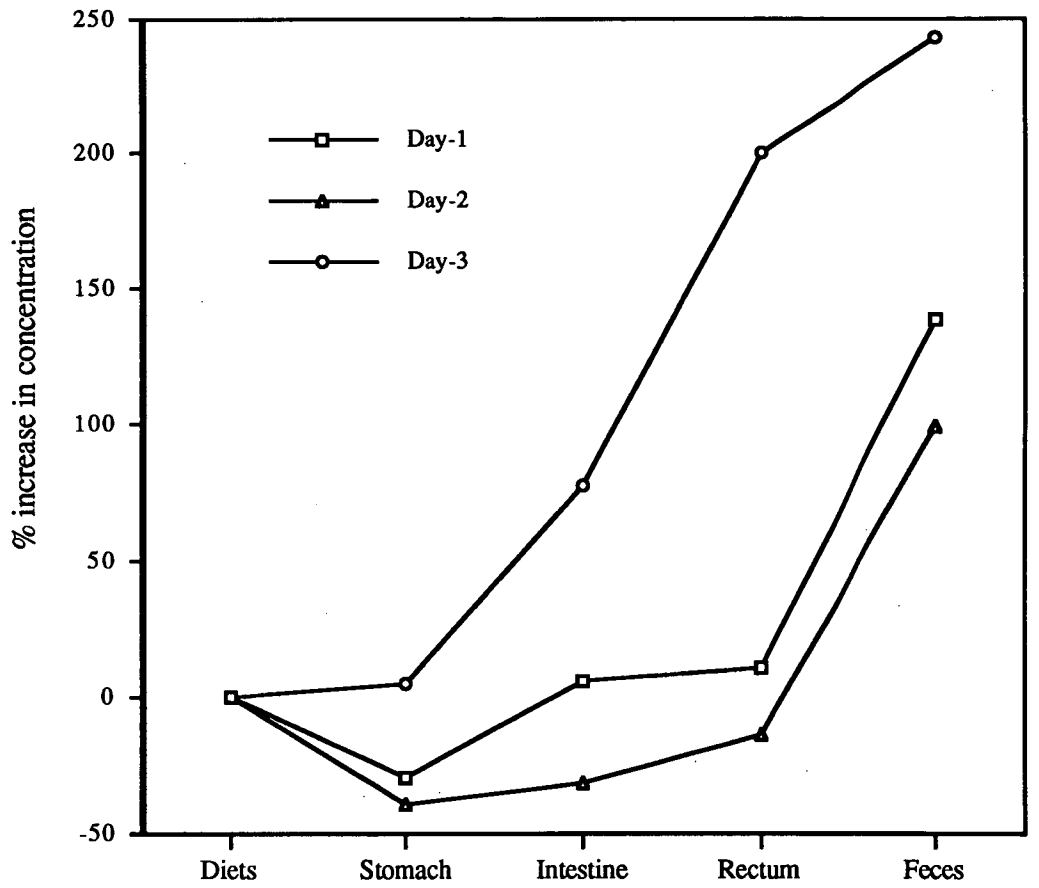


Figure 3.2.8 : Percentage increase of microtracer F-Ni at different parts of the digestive tract of rainbow trout fed experimental diet with 2.00% (w/w) microtracer F-Ni, as compared with the concentration of this marker in diet.

The results indicated that at 0.25 and 0.50% inclusion levels the percentage increase of microtracer F-Ni in the stomach and intestine were comparatively lower than rectum, where the increase becomes abruptly very high. On the other hand, the concentration in the feces remains much lower than the concentration of rectum.

At 1.00 and 2.00% inclusion levels, the percentage increase of microtracer concentration in all the parts of digestive tract were linear and progressive. At 2.00% inclusion level, although it was progressive, but the percentage increase in rectum were much lower than feces. The pattern of increase also were not consistent in all the days at 2% inclusion level. Another observation was the increased concentration in feces than rectum at 1.00 and 2.00% inclusion level which was contrary with 0.25 and 0.50 % inclusions.

3.2.4 Discussion

It is evident from the experiment that at 0.25 and 0.5% inclusion level microtracer F-Ni gave a erratic, inconsistent results. In this two inclusion the concentration in the feces remained much lower than rectum content. Theoretically, these concentrations should be the same or higher. In this experiment the whole rectum content were collected. Austeng (1978) reported that digestion occurred up to the distal end of the rectum. This finding suggests that as digestion occurred in the rectum, the concentration of marker should be increased in feces than rectum. With this point of view, the findings for 0.25 and 0.50% inclusion would not be acceptable. Moreover the pattern for the percentage increase over the three days of collection were not consistent.

At 2% level the increase was progressive but not sequential. The concentration in feces were much higher than rectum. The percentage increase in stomach and intestine in comparison with diet were either negative or very close to negative except for day 3. Low recovery from the digestive tract and at the same time high recovery from the feces suggests that the tracer moved faster through the digestive tract. Similar differential flow rate of chromic oxide has been observed in rainbow trout at 2% (w/w) inclusion level (Tacon and Rodrigues, 1984).

The recovery pattern for 1% inclusion level were quite promising. The rate of increase in concentration of marker tested in different parts of the digestive tract and feces were sequential and progressive for all the days.

In this experiment, no direct measurement for the passage rate and kinetics of food through the digestive tract or nutrient level in digesta were measured and compared. Therefore, it was not possible to calculate the digestibility of digesta obtained from the different parts of the digestive tract. Austreng (1978) measured digestibility at various parts of the gut and showed trends similar with those obtained in the present study with 1% inclusion level. The findings so far obtained in this study gave us some indication that at 1% inclusion level, the movement of microtracer F-Ni through the digestive tract would be uniform with the digesta. Therefore 1% inclusion of microtracer F-Ni into the diet have been selected for further digestibility studies.

Chapter Three

Experiment Three

Estimation of different digestibility coefficients using Microtracer F-Ni and comparison of these values with other markers

3.3.1 Introduction

During the passage of food through the digestive tract, not all of it is digested and absorbed. The undigested portion is egested as feces. The absorbed portion is determined by difference between the ingested and egested nutrients, and usually expressed as the percentage of the amount ingested as 'apparent digestibility coefficient'. Determination of digestibility coefficient by the direct or balance method is commonly used for many farm animals. Since the end of the last century it has been used for fish (Homburger, 1877, as cited by Hepher, 1988). The balance method is based on as accurate as possible determinations of the amounts of ingested and egested nutrients. However, with fish the food is given in water and feces are exerted into water, and nutrients of both food and feces may be dispersed and dissolved in water. Since the lost nutrients are treated as if they are absorbed by the fish, an error is caused in the digestibility coefficient which is biased towards higher values, according to the losses of nutrients in water.

There are thus two problems with direct methods of digestibility estimation in fish : one is to quantify the amount of food presented and amount of feces egested; another one is to quantify the loss of nutrients from feces when it remained in water. Windell et al. (1978) determined the effect of the time, the feces remain in water before they are collected, on the loss of nutrients, and found that the major loss is incurred during the first hours of immersion. During this time about 21% of the dry matter, 12% of the protein and 4% of the lipids were lost, increasing the digestibility coefficients by 11.5, 10 and 3.7 percentages respectively and within 16 hr the losses of

nutrients from the feces reached 31, 12 and 9.8% respectively, and the increase in digestibility coefficients were 17, 10 and 8.2% respectively.

In order to overcome the drawbacks of the direct / balance method, many researchers have been using the 'inert indicator' method. In this method a certain amount of inert substances, which is not digested by the fish, is introduced into the examined food. The concentrations of the nutrient and the inert indicator are determined in both the food and the feces and the apparent digestibility coefficient of the nutrient is calculated according to the formula described by Maynard and Loosli (1969). The advantage of this method over the previous one is that there is no need to determine the amount of food ingested nor the amount of feces egested. It is sufficient to analyse a sample of both and determine the percentage contents of nutrient and indicator. Nevertheless, even this method is not free of constraints and drawbacks. If the feces are to be sampled from water, after being egested, the analysis is subject to the same errors as the balance method due to dispersion and solution of nutrients in water.

To overcome the nutrient leaching problem from the feces after egestion different types of fecal collection system have employed by different authors (Austreng, 1978; De la Noüe and Choubert, 1986; Vens-Cappell, 1985, Cho and Slinger, 1979) to minimise this sort of error.

Aspects of basic methodology for digestibility studies were recently reviewed by Cho et al. (1985) and merits and demerits of the use of different markers (both external and internal) were reviewed by De

Silva (1989), Atkinson et al. (1984) amongst others (discussed in Chapter 1).

Although a number of markers have been in use for fish digestibility studies, there is still a controversy as to whether internal or external indicators are more suitable and / or reliable and which specific ones are to be used in digestibility estimations in fish (De Silva, 1989). Most of the markers have been used / studied singly as a tracer component in diet. Comparisons of more than one marker (external and internal) in the same feed has been rarely made (Buddington, 1980; Bowen, 1981; Tacon and Rodrigues, 1984) and as such validity of certain markers in digestibility studies remains unsettled (De Silva and Perera, 1983). Moreover, variability in digestion estimates and lack of agreement of digestion estimates from different markers have discouraged many researchers (Owens and Hanson, 1992).

Few works have been reported in using different markers in the same diet to compare the digestibility coefficients and most of them are contradictory. Tacon and Rodrigues (1984) compared the use of three external dietary markers (chromic oxide, polyethylene and acid-washed sand) and a natural internal dietary marker (crude fibre) for the estimation of apparent nutrient digestibility in rainbow trout. They incorporated all the external markers in the same diet but with various concentrations (0.5, 1.0 and 2.0% respectively) and found that dry matter, organic matter, crude protein and ash digestibility coefficients using chromic oxide, polyethylene (both at 1% inclusion level) and crude fibre as markers were not significantly different ($P > 0.05$) but differed with using AIA ($P < 0.05$) as an indicator. On the other hand

Atkinson et al. (1984) reported that apparent digestibilities of dry matter, crude protein and gross energy in a practical diet fed to rainbow trout (*Salmo gairdneri*) showed no significant difference ($P > 0.01$) using AIA and chromic oxide indicators. The only difference between Tacon and Rodrigues's (1984) and Atkinson et al's. (1984) was that the former included all the markers in the same diet and the later incorporated chromic oxide into one diet, celite into another diet and a third diet without external marker (for AIA determination), although the basic diet formulation were similar. De Silva and Perera (1983) compared the digestibility coefficients using three internal markers (crude fibre, hydrolysis resistant organic matter, and hydrolysis resistant ash) in the same diet fed to the Cichlid (*Etroplus suratensis*). They recommended HROM as a more reliable indigenous marker than either crude fibre or hydrolysis resistant ash.

In this present experiment two separate investigations (Trial 1 and Trial 2) were carried out to evaluate the suitability of microtracer F-Ni as an external marker in comparison with other commonly used markers such as chromic oxide and acid insoluble ash. In addition, the various effects of fecal collection methods and time of collection on digestibility coefficients and the effects of different incorporation of markers into the diets on digestibility coefficients were also evaluated in these trials.

In Trial 1, apparent digestibility coefficients of protein and dry matter were determined using microtracer F-Ni, chromic oxide and acid insoluble ash as markers incorporating 1% microtracer F-Ni and 1% chromic oxide separately into diets. Two time intervals were selected for fecal collection, 6 hour (09.30 - 15.30) period or a 24

hour (09.30 - 09.30 next day) period. Within each period feces were collected from fecal collection chamber or by stripping at the time of collection from fecal chamber.

In Trial 2 the experimental design was same as Trial 1 with the exception that an additional diet containing both 1% microtracer F-Ni and 1% chromic oxide were used to determine the ADC values. The objective was to determine and compare the ADC values from the same diet, fed to same fish, using same feces sample with various markers.

3.3.2 Materials and Methods

3.3.2.1 Diets

Trial 1

Two experimental diets were prepared according to the methods described in Experiment 2 using commercial trout pellet (Gibson Feed Mill, Tasmania) and mixed with microtracer F-Ni at 1% and chromic oxide at 1% (on air dry basis) levels respectively.

Trial 2

Three experimental diets were prepared according to the methods described in Experiment 2 using commercial trout pellet (Gibson Feed Mill, Tasmania) and incorporating 1% microtracer F-Ni, 1% chromic oxide and 1% chromic oxide and 1% microtracer F-Ni (on air dry basis) respectively.

3.3.2.2 Fish and the experimental system

Trial 1

Rainbow trout (*O. mykiss*) with an average initial weight 70.74 g (standard deviation 27.71 g.) were collected from National Key Centre for Aquaculture, University of Tasmania and distributed randomly between the tanks at a stocking density of 25 fish per tank. They were acclimatised in the tanks for a week before commencement of experiment. During that time they were fed with commercial trout pellet (Gibson Feed Mill, Tasmania). All of the 12 cylindro-conical fibreglass tanks, as described in Chapter 2, were

used for this experiment with six tanks randomly allocated for each treatment. The tanks were provided with natural day light / dark period conditions and the water temperature varied from 7.8°C to 11.5°C during the experimental period of 15 days.

Trial 2

Rainbow trout (*O. mykiss*) with average initial weight 103.37 g. (standard deviation 27.31 g.) were collected from National Key Centre for Aquaculture, University of Tasmania and they were distributed randomly between the tanks at a stocking density of 25 fish per tank and their initial and final weight were recorded. They were acclimatised in the tanks for a week before experimental feeding commenced. During that time they were fed commercial trout pellet (Gibson Feed Mill, Tasmania). All of the 12 cylindroconical fibreglass tanks, as described in Chapter 2, were used in this experiment arranged four tanks for each treatment randomly. Four tanks were allocated for each treatment, two tanks were selected randomly for 6 hours fecal collection and the remaining two for 24 hours fecal collection. The water temperature varied from 8°C to 12.8°C during the experimental period of 15 days. The tanks were provided with natural day light / dark period conditions.

3.3.2.3 Feeding and fecal collection

Trial 1

The fish were fed once a day at 0900 h with experimental diets *ad libitum*. The fecal collectors were thoroughly cleaned with a brush

half an hour after feeding to remove the uneaten feed, fecal residues and other extraneous organic matter.

Two time intervals and two fecal collection procedures were selected. Feces were allowed to settled over a 6 h period (09.30 - 15.30) or a 24 h period (excluding initial 6 h fecal deposition period) i.e., 09.30 - 09.30 next day. Feces were collected from each of the fecal collector for 6 h or 24 h after the collectors were thoroughly cleaned. At the time, the feces were sampled from the collector (6 h or 24 h post cleaning), 10 fish from each tank were sampled randomly, anaesthetised with benzocaine (ethyl 4-amino benzoate) at a concentration of 40 mg per litre. Surface water on the fish was removed with a paper towel. With the left hand holding the fish's head, using the forefinger and thumb of the right hand, a gentle pressure was exerted on the abdomen moving along the lateral line system towards the anus. By this method some urine could be pressed out, reducing the risk of contaminating the feces later (Vens-Cappell, 1985). Then repeating the similar movement, with greater pressure on the region between anal fin and the anus, feces was stripped onto a pre-weighed dry Whatman filter paper. Water and urine stripped with feces were thus absorbed immediately from feces. Immediately after stripping, the fish were returned to their respective tanks.

Collection of feces was started on the third day after commencement of feeding (De la Noüe et al., 1980) and were followed by collection on the 5th, 7th and 14th days. All fecal samples were dried in the oven at 80°C for 24 hours, ground and stored in a desiccator for subsequent chemical analyses. As the collected samples from stripping were very small to run all the analytical procedures,

samples from three tanks were pooled into one sample and those from other three tank were pooled to provide the other duplicate. The corresponding fecal samples were also pooled.

Trial 2

The fish were fed once a day at 0900 hours with experimental diets to satiation and the amount of diet consumed per tank per day were recorded for feed conversion efficiency analyses. The fecal collectors were cleaned with a brush 30 minute after feeding to remove the uneaten feed, fecal residues and often extraneous organic matter.

The same fecal collection procedure of Trial 1 were followed with the exception that, feces were sampled at 6 and 24 hours collection but stripping of feces only at 24 hours collection after feeding.

3.3.2.4 Analytical procedure

The microtracer particles were recovered by using the magnetic wand, developed for counting as the methods described in Chapter 2, and the recovery (% w/w) were calculated.

Ash content were determined by placing the sample into a furnace at 550^{0}C for 6 hours. Using the ash residues, acid insoluble ash (AIA) were determined by treating the residues with acid and re-ashing at 550^{0}C for 2 hours (AOAC, 1980).

Nitrogen was determined using the LECO Elemental Analyser (model CHNS-932) as described by Anon (1991). Samples being analysed were exposed to a high temperature oxygen atmosphere in

the combustion furnace within the analyser, which allowed the material to combust, releasing nitrogen as nitrogen oxide. This nitrogen oxide was reduced back to nitrogen by Cu catalyst and delivered to a highly sensitised thermal conductivity detection system for measurement. In this method, control of the analysis from automatic weight recording of the sample through the printing of the final result were performed by a 16-bit microprocessor system. Crude protein content of the samples were calculated by multiplying the total nitrogen (N) within the sample with an empirical factor 6.25. This factor is based on the assumption that the average protein contains about 16% N. In practice, however, a variation of between 12%-19% is possible between individual proteins (Tacon, 1990).

Chromium content in the experimental diets and feces were determined photometrically Anon (1989) after acid digestion method Vogel (1972) as follows :

A known amount (0.1 g.) of sample were fused with sodium peroxide (2.5 - 3.0 g.) and sodium carbonate (0.5 g.) in a iron-nickel crucible. This was carried out by placing the crucible over a Bunsen burner till no visible reaction occurred and then red heated for 10 minutes and allowed to cool. Then the crucible were placed into a mixture of 50 ml of water and 25 ml of concentrated (3.5 N) HCl. This dissolution were heated to melt and then the crucible were removed after washing. The solution were filtered into a 100 ml volumetric flask. Chromium content were determined by flame atomic absorption spectrometer (model Spectr. AA 300) with 1 -100 standards. Chromium were reported as Cr_2O_3 by multiplying the chromium value with the factor 1.46 (considering the proportion of the molecular weight of chromium in Cr_2O_3).

3.3.2.5 Digestibility calculations

Apparent protein digestibility coefficients were determined using the formula of Maynard and Loosli (1969).

$$ADC\ (\%) = 100 \times \left(1 - \frac{(\%\ \text{marker in diet})}{(\%\ \text{marker in feces})} \times \frac{\%\ \text{protein in feces}}{\%\ \text{protein in diets}} \right).$$

Apparent dry matter digestibility coefficient were determined using the formula of Barash et al. (1983).

$$ADC\ (\%) = 100 \times \left(1 - \frac{\%\ \text{marker in diet}}{\%\ \text{marker in feces}} \right).$$

Considering the small quantity of samples, the stripped fecal contents were analysed for chromic oxide, protein and microtracers only. The others were also analysed for acid insoluble ash in addition to the above mentioned chemical parameters.

Specific growth rate (SGR) and food conversion ratio (FCR) were calculated by the following formula (Wee, 1983) :

$$SGR\ (\%) = \frac{\text{Ln of final body weight - Ln of initial body weight.}}{\text{Time (days)}} X100.$$

$$FCR\ (\%) = \frac{\text{Weight of food presented (dry weight)}}{\text{Weight of fish produced (fresh weight)}}.$$

3.3.2.6 Statistical analyses

The apparent digestibility coefficient values of protein and dry matter estimated with two methods (collection from fecal chamber and by stripping) and two different times (6 hours and 24 hours) of fecal collection using two different markers (microtracer F-Ni and chromic oxide) over four different days of fecal collection (Trial 1) were analysed by 4 way ANOVA.

The apparent digestibility coefficients of protein and dry matter using only fecal collections from fecal collection chamber with microtracer F-Ni, chromic oxide, acid insoluble ash (from microtracer containing diet) and acid insoluble ash (from chromic oxide containing diet) as markers and feces collected at 6 and 24 hours over four different days during the experiment (Trial 1)were subjected to 3 way ANOVA.

The apparent digestibility coefficients (%) of protein and dry matter estimated on feces collected with 3 methods and times (6 h fecal collection, 24 h fecal collection and 24 h stripping) over four different days of fecal collection with two markers (microtracer F-Ni and chromic oxide) incorporated either singly or both in the experimental diets in Trial 2 were analysed by a 4 way ANOVA.

The ADC (%) of protein and dry matter were estimated using feces collected at 6 h and 24 h after feeding from fecal collection chamber over four different days with seven resulting markers (microtracer F-Ni, chromic oxide, AIA from microtracer containing diet, AIA from Cr_2O_3 containing diet - all from single marker inclusion, and microtracer F-Ni, Cr_2O_3 and AIA from double marker inclusion).

These data were analysed by a 3 way ANOVA (7 markers x 4 days x 2 collection times.

Results were considered to be statistically significant if $P < 0.05$. Prior to performing ANOVA, normality of the data were determined using Shapiro-Wilk W test and homogeneity of the variances was determined using Bartlett's test and Cochran's test. If the variances were heterogeneous appropriate transformation of the data were made. When the obtained P value for the treatment or interaction in ANOVA were significant, determination of which pairs of groups were significant was made by multiple comparison of means using Fisher's LSD (Sokal and Rohlf, 1987). All the tests except Cochran's were made using JMP 2.0 computer package.

3.3.3 Results

3.3.3.1 Composition of diets and feces

Trial 1

The contents of crude protein (N x 6.25), ash and different markers of experimental diets and feces are shown in Table 3.3.3.1 and 3.3.3.2 respectively. Despite of the fact that the experimental diets have the same composition, the ash content in microtracer containing diet were 9.41% higher than that of chromic oxide containing diet. The acid insoluble ash content were 202.2% higher in the microtracer containing diet than chromic oxide containing diet. A similar trend with increased amount of ash and acid insoluble ash in the feces produced by fish fed diets with microtracer F-Ni were also observed (Table 3.3.3.2).

Trial 2

The contents of crude protein (N x 6.25), ash and different markers of experimental diets and feces are shown in Tables 3.3.3.3 and 3.3.3.4. Despite the use of same basic diet to incorporate microtracer F-Ni and chromic oxide in preparation of three experimental diets, the ash content in diet with microtracer F-Ni and diet with both marker were 10.67 and 18.37 percents higher than that of chromic oxide containing diets. Similarly the acid insoluble ash contents of the same were 254.55 and 318.18 percents higher than chromic oxide containing diet. A similar trend with increased amount of ash and acid insoluble ash in the feces produced by fish fed diets with microtracer F-Ni and both microtracer F-Ni and chromic oxide were also observed (Table 3.3.3.4).

Table 3.3.3.1 : Crude protein (N x 6.25), ash and marker contents in diets (mean ± SE, n=3, expressed as % w/w on a moisture free basis) in Trial 1.

Components	Diets	
	with microtracer	with chromic oxide
Crude protein	52.17 ± 1.09	50.00 ± 0.32
Ash	10.23 ± 0.11	9.35 ± 0.08
Chromic oxide	-	1.27 ± 0.13
Microtracer F-Ni	0.92 ± 0.07	-
Acid insoluble ash	1.48 ± 0.02	0.49 ± 0.03

Table 3.3.3.2 : Crude protein (N x 6.25), ash and marker contents of feces collected over 14 days feeding period (Trial 1) for chromic oxide and microtracer F-Ni containing diets (mean with SE in parentheses; n = 8; expressed as % w/w on a moisture free basis).

Components	Feces from microtracer containing diet				Feces from Cr_2O_3 containing diet			
	6 h. collection		24 h. collection		6 h. collection		24 h. collection	
	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber
Crude protein	17.45 (0.16)	15.74 (0.31)	14.68 (0.30)	14.74 (0.40)	15.82 (0.41)	15.36 (0.45)	15.51 (0.45)	15.06 (0.39)
Ash	-	28.28 (0.70)	-	26.76 (0.74)	-	22.13 (0.39)	-	22.47 (0.62)
Acid insoluble ash	-	6.79 (0.74)	-	5.90 (0.46)	-	3.63 (0.26)	-	3.77 (0.31)
Microtracer F-Ni	2.45 (0.20)	2.65 (0.21)	2.19 (0.15)	3.38 (0.19)	-	-	-	-
Chromic oxide	-	-	-	-	3.20 (0.24)	3.68 (0.14)	3.11 (0.08)	4.44 (0.11)

Table 3.3.3.3 : Crude protein (N x 6.25), ash and marker contents in diets (mean expressed as % w/w on a moisture free basis) in Trial 2.

Components	Diets		
	with micro -tracer F-Ni	with chromic oxide	with Cr_2O_3 and microtracer
Crude protein	48.29	47.86	47.90
Ash	10.06	9.09	10.76
Chromic oxide	-	1.63	1.60
Microtracer F-Ni	1.20	-	1.16
Acid insoluble ash	1.17	0.33	1.38

Table 3.3.3.4 : Crude protein (N x 6.25), ash and marker contents of feces collected over 14 days feeding period (in Trial 2) for the experimental diets (means with SE in the parentheses, n = 8, expressed as % w/w on a moisture free basis).

Components	Feces from microtracer containing diet			Feces from chromic oxide containing diet			Feces from both microtracer and Cr_2O_3 containing diet		
	6 h F[1]	24 h S[2]	24 h F	6 h F	24 h S	24 h F	6 h F	24 h S	24 h F
Crude protein	15.82 (1.68)	17.56 (0.50)	16.16 (0.60)	15.28 (0.22)	17.60 (0.34)	14.60 (0.28)	16.66 (0.47)	17.72 (0.47)	15.47 (0.47)
Ash	24.46 (0.69)	-	23.76 (0.39)	21.83 (0.26)	-	21.41 (0.37)	29.68 (0.47)	-	28.77 (0.45)
Acid insoluble ash	2.71 (0.24)	-	2.92 (0.23)	2.32 (0.12)	-	2.74 (0.31)	4.67 (0.51)	-	5.92 (0.35)
Microtracer F-Ni	2.55 (0.51)	2.54 (0.70)	3.21 (0.10)	-	-	-	2.23 (0.08)	2.60 (0.45)	3.66 (0.53)
Chromic oxide	-	-	-	3.61 (0.14)	3.75 (0.10)	4.43 (0.15)	3.85 (0.11)	3.88 (0.09)	4.56 (0.14)

[1]denotes collection of feces from fecal chamber and [2]denotes collection of feces by stripping.

3.3.3.2 Apparent digestibility coefficients of protein

Trial 1

The apparent digestibility coefficients (%) of protein in diets estimated using feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding over four different days of collection using microtracer F-Ni and chromic oxide as markers during the experiment is shown in Table 3.3.3.5 and the analysis of variance for the same is shown in Table 3.3.3.6. The overall ADC (%) of protein using the above two markers over two collection methods and two different times of fecal collection is shown in Figure 3.3.3.1. Fecal collection days and the use of markers, microtracer F-Ni and chromic oxide, showed no significant effect on ADC of protein values ($P > 0.05$). Time of fecal collection ie., feces collected over 24 hour generated higher ADC than 6 hour collection. Methods of fecal collection ie., collection from fecal chamber generated significantly higher ($P < 0.001$) protein digestibility values than stripped samples. But ADC calculated from stripped fecal samples at 6 or 24 hours after feeding were not significantly different ($P > 0.05$). None of the interactions between the factors were significantly ($P > 0.05$) different (Table 3.3.3.6).

The apparent digestibility coefficients (%) of protein in diets estimated from feces collected from fecal collection chamber at 6 and 24 fours interval of feeding over four different days of collection using microtracer F-Ni, chromic oxide and two sources of acid insoluble ash as markers is shown in Table 3.3.3.7 and the analysis of variance for the same in Table 3.3.3.8. The overall ADC

(%) of protein using the above four markers over two different times of fecal collection from fecal collection chamber is shown in Figure 3.3.3.2. Fecal collection days showed no significant effect on ADC of protein values ($P > 0.05$) for all four markers used. Markers used however generated significantly ($P< 0.0001$) different ADC of protein values. Apparent digestibility coefficient of protein values calculated using acid insoluble ash markers were significantly higher than that using microtracer F-Ni or chromic oxide markers. The ADC of protein estimated using two sources of acid insoluble ash differed significantly ($P<0.05$) amongst themselves with ADC using microtracer containing diet were higher than ADC from chromic oxide containing diet. However there were no significant differences ($P>0.05$) between the ADC of protein calculated from 6 h and 24 h fecal collection method with AIA markers.

Time of fecal collection ie., 6 h or 24 h after feeding was also a significant factor ($P < 0.05$) with 24 hour collection generated higher ADC values than 6 hour collection (Table 3.3.3.7). None of the interactions between the factors were significant ($P > 0.05$). The multiple comparison of means for the ADC of protein in diets estimated using four different markers with two methods of fecal collection and two different times of collection is shown in Table 3.3.3.9.

Table 3.3.3.5 : The apparent digestibility coefficients (%) of protein (mean with s.e.m. in parentheses, n = 2) in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding over four different days of collection using microtracer F-Ni and chromic oxide as markers in Trial 1.

Days	Microtracer F-Ni marker				Chromic oxide marker			
	6 hours collection		24 hours collection		6 hours collection		24 hours collection	
	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber
3	87.52	88.23	87.65	93.13	85.84	89.32	85.88	91.55
	(2.08)	(3.95)	(2.57)	(0.26)	(0.21)	(0.03)	(1.69)	(0.43)
5	84.57	89.00	87.05	92.62	88.94	89.93	87.60	90.98
	(4.94)	(1.81)	(1.08)	(0.53)	(0.68)	(1.20)	(0.69)	(0.27)
7	86.06	90.14	90.22	92.47	83.00	89.17	88.50	90.39
	(0.39)	(2.61)	(0.24)	(0.99)	(0.07)	(0.22)	(0.12)	(0.28)
14	88.91	88.62	86.19	90.40	90.12	89.17	87.28	92.62
	(1.87)	(0.10)	(2.55)	(0.94)	(0.31)	(0.11)	(0.60)	(0.03)
Total mean	86.76	89.00	87.77	92.17	86.97	89.40	87.31	91.38
	(1.24)	(0.99)	(0.91)	(0.47)	(1.06)	(0.26)	(0.51)	(0.33)

Table 3.3.3.6 : Analysis of variance for the apparent digestibility coefficients (%) of protein in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding over four different days using microtracer F-Ni and chromic oxide as markers.

Sources of variation	DF	SS	MS	F	P
Day (A)	3	2.5682	0.8561	0.1696	0.9161
Marker (B)	1	0.4160	0.4160	0.0824	0.7759
Time (C)	1	42.445	42.445	8.4104	0.0067*
Collection Method (D)	1	172.13	172.13	34.1078	0.0000*
A x B	3	29.433	9.8110	1.9440	0.1423
A x C	3	22.847	7.6156	1.5090	0.2310
A x D	3	7.6106	2.5369	0.5027	0.6831
B x C	1	3.4689	3.4689	0.6874	0.4132
B x D	1	0.0203	0.0203	0.0040	0.9498
C x D	1	14.535	14.535	2.8801	0.0994
A x B x C	3	9.1714	3.0571	0.6058	0.6161
A x B x D	3	10.910	3.6366	0.7206	0.5471
A x C x D	3	39.549	13.183	2.6122	0.0683
B x C x D	1	0.2756	0.2756	0.0546	0.8167
A x B x C x D	3	4.0063	1.3354	0.2646	0.8504
Error	32	161.50	5.0468		
Total	63	520.89			

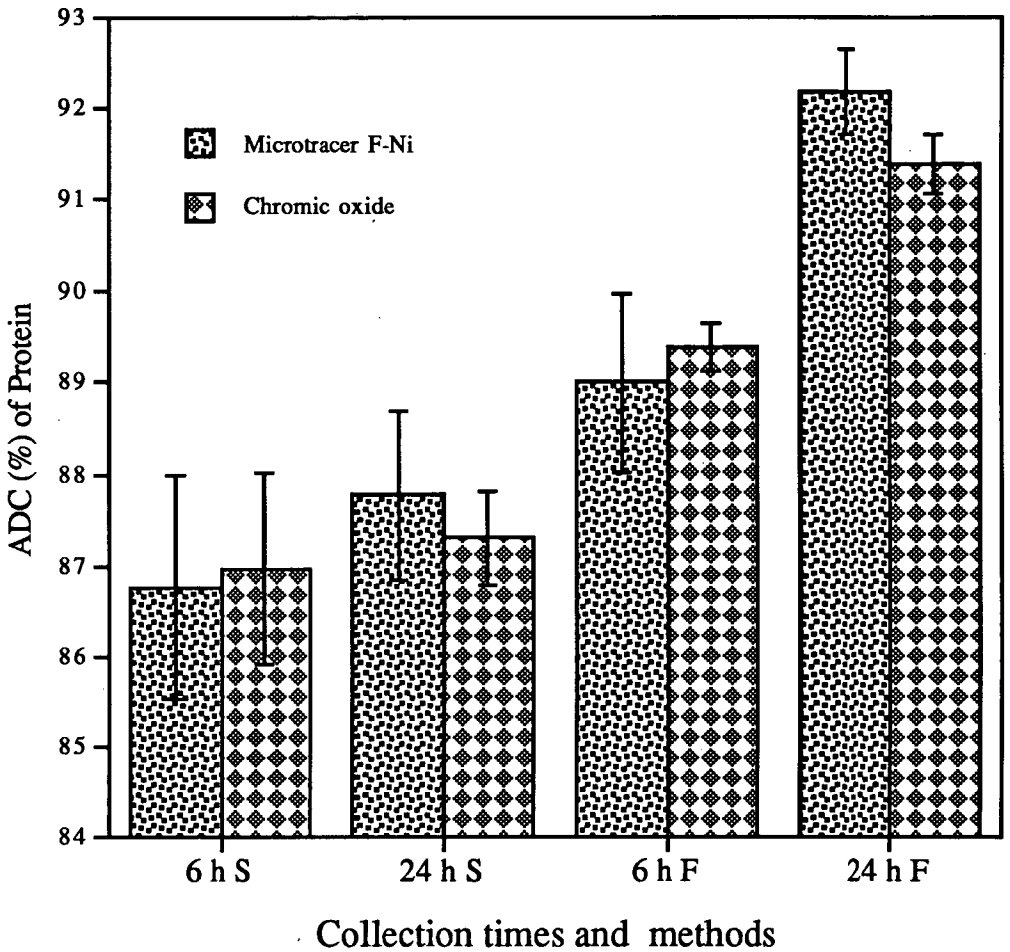


Figure 3.3.3.1 : The overall apparent digestibility coefficients (%) of protein (mean ± SE, n = 8 considering days as replication) in diets estimated on feces collected from fecal collection chamber (F) and by stripping (S) and 6 and 24 hours interval of feeding using microtracer F-Ni and chromic oxide as markers.

Table 3.3.3.7 :The apparent digestibility coefficients (%) of protein (mean with s.e.m. in parentheses, n = 2) in diets estimated on feces collected from fecal collection chamber at 6 and 24 hours interval of feeding over four different days of collection using microtracer F-Ni, chromic oxide and acid insoluble ash (AIA) as markers in Trial 1.

Days	Microtracer F-Ni marker		Chromic oxide marker		AIA marker (from microtracer - diet)		AIA marker (from Cr_2O_3 - diet)	
	6 hours collection	24 hours collection	6 hours collection	24 hours collection	6 hours collection	24 hours collection	6 hours collection	24 hours collection
3	88.23	93.13	89.32	91.55	92.01	91.35	95.50	96.77
	(3.95)	(0.26)	(0.03)	(0.43)	(3.64)	(0.74)	(0.35)	(0.22)
5	89.00	92.62	89.93	90.98	92.94	93.49	95.80	95.60
	(1.81)	(0.53)	(1.20)	(0.27)	(1.55)	(0.08)	(0.69)	(0.70)
7	90.14	92.47	89.17	90.39	92.61	93.66	96.06	95.54
	(2.61)	(0.99)	(0.22)	(0.28)	(2.48)	(0.00)	(0.74)	(1.07)
14	88.62	90.49	89.17	92.62	93.55	92.75	95.67	95.64
	(0.10)	(0.94)	(0.11)	(0.03)	(0.53)	(2.06)	(0.76)	(1.31)
Total mean	89.00	92.17	89.40	91.38	92.78	92.69	95.75	95.88
	(0.99)	(0.47)	(0.26)	(0.33)	(1.06)	(0.72)	(0.26)	(0.40)

Table 3.3.3.8 : Analysis of variance for the apparent digestibility coefficients (%) of protein in diets estimated on feces collected from fecal collection chamber at 6 and 24 hours interval of feeding over four different days using microtracer F-Ni, chromic oxide and two sources of acid insoluble ash as indicators.

Sources of variation	DF	SS	MS	F	P
Day	3	1.0980	0.3660	0.0948	0.9624
Marker	3	307.02	102.34	26.497	0.0000*
Time	1	28.396	28.396	7.3521	0.0107*
Day x Marker	9	15.251	1.6945	0.4387	0.9036
Day x Time	3	2.0376	0.6792	0.1759	0.9120
Marker x Time	3	27.856	9.2852	2.4041	0.0857
Day x Marker x Time	9	11.567	1.2853	0.3328	0.9574
Error	32	123.59	3.8622		
Total	63	516.81			

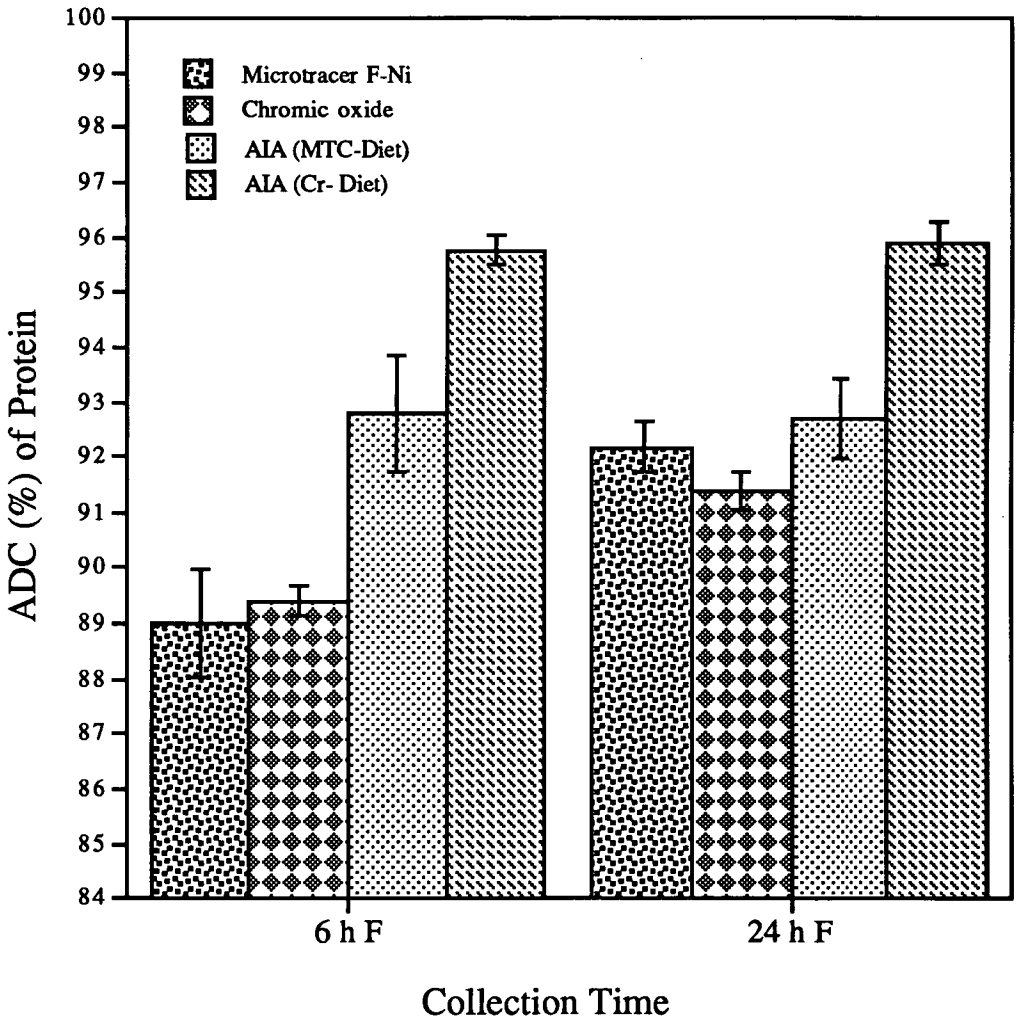


Figure 3.3.3.2 : The overall apparent digestibility coefficients (%) of protein (mean ± SE., n = 8 considering days as replication) in diets estimated on feces collected from fecal collection chamber (F) at 6 and 24 hours of interval after feeding using four different markers.

Table 3.3.3.9: Multiple comparison of means for the apparent digestibility coefficients (%) of protein in Trial 1 (mean with SE in the parentheses, n = 8, considering days as replication) in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding by four different markers.

Markers	6 hours collection		24 hours collection	
	Stripping	Fecal chamber	Stripping	Fecal chamber
Microtracer F-Ni	$_{e}$ 86.76 ^{a} (1.24)	$_{e}$ 89.00 ^{a} (0.99)	$_{e}$ 87.77 ^{a} (0.91)	$_{ef}$ 92.17 ^{b} (0.47)
Chromic oxide	$_{e}$ 86.97 ^{a} (1.06)	$_{e}$ 89.40 ^{b} (0.26)	$_{e}$ 87.31 ^{a} (0.51)	$_{e}$ 91.38 ^{c} (0.33)
AIA (MTC)[1]	-	$_{f}$ 92.78 ^{a} (1.06)	-	$_{f}$ 92.69 ^{a} (0.72)
AIA (Cr_2O_3)[2]	-	$_{g}$ 95.75 ^{a} (0.26)	-	$_{g}$ 95.88 ^{a} (0.40)

[1]Acid insoluble ash as a marker from microtracer F-Ni containing diets and feces.

[2]Acid insoluble ash as a marker from Cr_2O_3 containing diets and feces.

$^{abc}/_{efg}$ Mean values of components with the same superscript in same row and same subscript in same column are not significantly different ($P > 0.05$).

Trial 2

The apparent digestibility coefficients (%) of protein in diets estimated on feces collected from fecal collection chamber at 6 and 24 h after feeding, over four different days of fecal collection, using diets with microtracer F-Ni, chromic oxide and both of them as markers is shown in Table 3.3.3.10 and the analysis of variance for the same is shown in Table 3.3.3.11. The overall ADC (%) of protein using the above two markers with two inclusion levels (single or both), using three collection times and methods (6 h fecal collection, 24 h fecal collection and 24 h stripping) is shown in Figure 3.3.3.3 and the multiple comparison of means for the same in Table 3.3.3.12(a). The effect of fecal collection days and the use of different markers on ADC of protein values were not significantly different ($P>0.05$). Inclusion of these two markers as single or both into experimental diets, as a whole, resulted a significant ($P > 0.05$) increase of ADC values in diets with both the markers. Time and method of fecal collection were highly significant ($P < 0.001$) with 24 h fecal collection had the highest ADC values. Although with the fecal samples of 24 h stripping resulted the lowest ADC values but it remained similar ($P > 0.05$) with the ADC values for 6 h fecal collection. Within the same time and collection method, ADC of protein values were higher for the diets containing both markers than the diets containing single marker but they were not significantly ($P > 0.05$) different. None of the interactions between the treatments were significant ($P > 0.05$).

The apparent digestibility coefficients of protein estimated on feces collected from fecal collection chamber at 6 h and 24 h after feeding

over four different days of fecal collection using seven resulted markers is shown in Table 3.3.3.13 and the analysis of variance and multiple comparison of means for the same in Tables 3.3.3.14 and 3.3.3.12(b). The overall ADC of protein using four markers with two different times of fecal collection at single inclusion level is shown in Figure 3.3.3.4. The ADC of protein using three sources of acid insoluble ash as markers is shown in Figure 3.3.3.5. The day of fecal collection were not significant ($P > 0.05$) at all. But the use of different markers appeared as highly significant ($P < 0.0001$). Among the different markers, microtracer F-Ni and chromic oxide were not significantly different from each other ($P > 0.05$) for the ADC of protein values. On the other hand, the ADC of protein values using acid insoluble ash (from chromic oxide and both marker containing diets) as markers remained significantly higher ($P < 0.05$) with the highest value for chromic oxide containing diets. Collection time and procedure were also highly significant ($P < 0.0001$) with 24 h fecal collection resulted the highest ADC values and differed significantly with 6 h fecal collection (for microtracer F-Ni and chromic oxide containing diets). The trends of apparent digestibility coefficients of protein with microtracer F-Ni and chromic oxide as markers were found similar ($P >0.05$) for 6 h and 24 h fecal collections with all the inclusions. Acid insoluble ash (from both microtracer and chromic oxide containing diets) resulted similar ($P > 0.05$) ADC of protein values for 6h and 24 h collections. ADC of protein with chromic oxide marker (single inclusion) at 6 h fecal collection differed significantly ($P < 0.05$) with that of double included marker.

Table 3.3.3.10 : The apparent digestibility coefficients (%) of protein (mean with SE in parentheses, n = 2) estimated on feces collected from fecal collection chamber at 6 and 24 hours and by stripping at 24 hours after feeding over four different days of fecal collection using diets with microtracer F-Ni (D -1), Cr_2O_3 (D - 2) and both of them (D - 3) as markers in Trial 2.

Day	6 h collection from fecal chamber				24 h collection from fecal chamber				24 h collection by stripping			
	D-1	D-2	D-3		D-1	D-2	D-3		D-1	D-2	D-3	
	MTC[1]	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3
3	84.35	84.10	85.52	85.58	85.89	88.50	90.30	88.90	83.76	85.94	85.63	84.94
	(2.00)	(0.32)	(0.36)	(0.81)	(3.85)	(0.41)	(1.02)	(0.98)	(2.52)	(0.64)	(0.68)	(0.88)
5	85.23	83.62	87.40	86.00	86.62	87.66	89.46	88.40	84.46	85.81	84.44	85.44
	(1.83)	(0.66)	(0.60)	(0.52)	(0.79)	(0.11)	(0.37)	(0.04)	(0.32)	(0.27)	(0.20)	(1.44)
7	81.15	85.70	85.46	86.34	89.54	88.40	88.33	88.20	80.33	83.70	84.36	84.27
	(1.28)	(0.43)	(3.08)	(0.52)	(0.54)	(0.78)	(0.38)	(1.45)	(0.24)	(0.07)	(1.45)	(0.45)
14	83.55	87.08	81.91	86.26	87.44	87.27	89.63	89.19	81.50	82.48	83.12	84.46
	(4.93)	(0.61)	(1.19)	(0.24)	(0.85)	(0.27)	(1.19)	(0.95)	(4.61)	(1.77)	(2.51)	(0.59)
Total Mean	83.57	85.12	85.07	86.05	87.37	87.95	89.43	88.67	82.51	84.48	84.38	84.78
	(1.42)	(0.55)	(1.14)	(0.24)	(1.07)	(0.26)	(0.48)	(0.40)	(1.36)	(0.66)	(0.76)	(0.39)

[1]MTC denotes microtracer F-Ni as a marker.

Table 3.3.3.11: Analysis of variance for the ADC (%) of protein estimated on feces collected from fecal collection chamber at 6 and 24 hours and by stripping at 24 hours after feeding over four different days of collection using microtracer F-Ni and chromic oxide as markers with single and both inclusion in experimental diets.

Sources of variation	DF	SS	MS	F	P
Day (A)	3	14.3157	4.7719	0.9983	0.4018
Marker (B)	1	14.8444	14.844	3.1055	0.0844
Inclusion (C) (single vs both)	1	36.1989	36.1989	7.5730	0.0083*
Collection procedure (D)	2	331.543	165.772	34.6799	0.0000*
A x B	3	10.8860	3.6286	0.7591	0.5226
A x C	3	1.0278	0.3426	0.0717	0.9748
A x D	6	24.3667	4.0611	0.8496	0.5384
B x C	1	8.1375	8.1375	1.7024	0.1982
B x D	2	9.1958	4.5979	0.9619	0.3894
C x D	2	0.3657	0.1828	0.0383	0.9625
A x B x C	3	6.2592	2.0864	0.4365	0.7229
A x B x D	6	29.111	4.8519	1.0150	0.4269
A x C x D	6	36.5508	6.0918	1.2744	0.2868
B x C x D	2	1.0948	0.5474	0.1145	0.8920
A x B x Cx D	6	12.6807	2.1134	0.4421	0.8469
Error	48	229.442	4.7800		
Total	95	766.02			

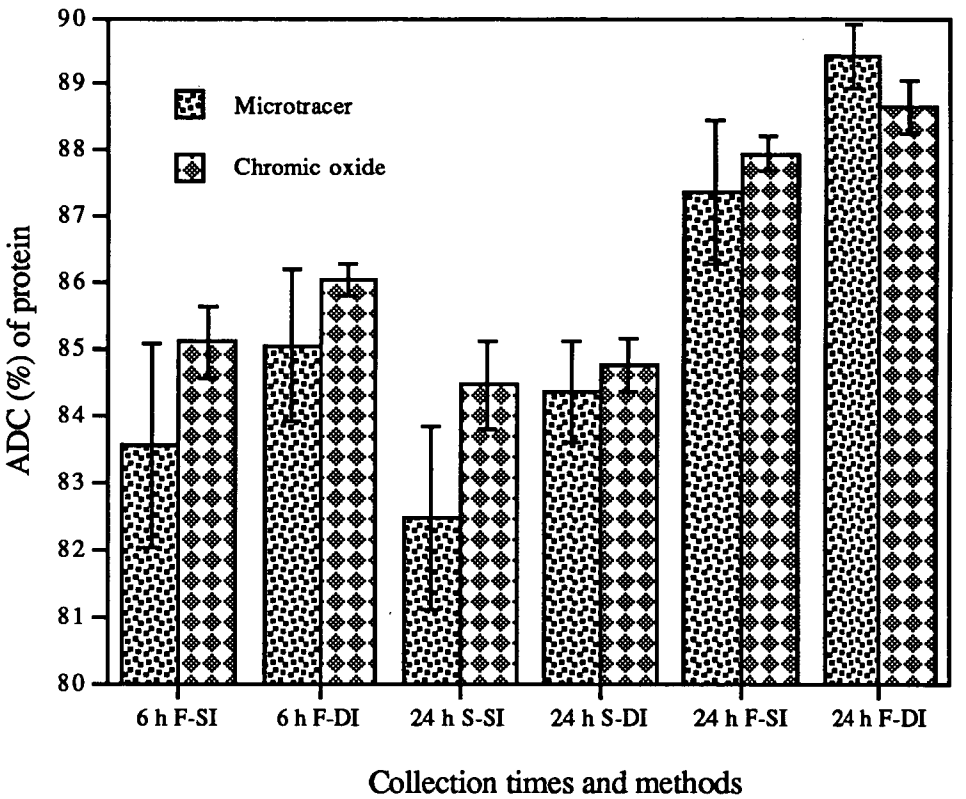


Figure 3.3.3.3 : The overall apparent digestibility coefficients (%) of protein (mean ± SE, n=8, considering days as replication) in diets estimated on feces collected from fecal collection chamber at 6 hours (6 h F) and 24 hours (24 h F) and by stripping at 24 hours (24 h S) after feeding using microtracer F-Ni and chromic oxide with two inclusion levels (single inclusion-SI, double inclusion-DI) as markers.

Table 3.3.3.12 (a) : Multiple comparison of means for the apparent digestibility coefficients (%) of protein (mean with SE in the parentheses, n = 8, considering days as replication) in diets estimated on feces collected at 6 and 24 hours after feeding using microtracer F-Ni and chromic oxide(included as single or both into diets) as markers in Trial 2 (ANOVA Table 3.3.3.11).

Markers	6 hours fecal collection		24 hours fecal collection		24 hours stripping	
	Single inclusion	Double inclusion	Single inclusion	Double inclusion	Single inclusion	Double inclusion
Microtracer F-Ni	$_{e}$ 83.57 ^{a} (1.52)	$_{e}$ 85.07 ^{ab} (1.14)	$_{e}$ 87.37 ^{bc} (1.07)	$_{e}$ 89.43 ^{c} (0.48)	$_{e}$ 82.51 ^{a} (1.36)	$_{e}$ 84.38 ^{a} (0.76)
Chromic oxide	$_{e}$ 85.12 ^{ab} (0.55)	$_{e}$ 86.05 ^{b} (0.24)	$_{e}$ 87.95 ^{c} (0.26)	$_{e}$ 88.67 ^{c} (0.40)	$_{e}$ 84.48 ^{a} (0.66)	$_{e}$ 84.78 ^{a} (0.39)

$^{abc}/_{e}$ Mean values of components with the same superscript in same row and same subscript in same column are not significantly different ($P > 0.05$).

Table 3.3.3.13 : The apparent digestibility coefficients (%) of protein (mean with SE in parentheses, n = 2) estimated on feces collected from fecal collection chamber at 6 and 24 hours after feeding over four different days of collection and using seven resulting markers.

Day	6 h collection from fecal chamber							24 h collection from fecal chamber						
	MTC (S)	Cr_2O_3 (S)	AIA (mtc-S)	AIA (Cr-S)	MTC (D)	Cr_2O_3 (D)	AIA (D)	MTC (S)	Cr_2O_3 (S)	AIA (mtc-S)	AIA (Cr-S)	MTC (D)	Cr_2O_3 (D)	AIA (D)
3	84.35	84.10	82.80	94.53	85.52	85.58	89.31	85.89	88.50	82.67	95.00	90.30	88.90	92.34
	(2.00)	(0.32)	(4.93)	(0.65)	(0.36)	(0.81)	(3.72)	(3.85)	(0.41)	(1.17)	(0.64)	(1.02)	(0.98)	(1.31)
5	85.23	83.62	86.19	94.71	87.40	86.00	90.28	86.62	87.66	88.61	95.25	89.46	88.40	92.90
	(1.83)	(0.66)	(0.11)	(0.62)	(0.60)	(0.52)	(0.13)	(0.79)	(0.11)	(0.15)	(0.64)	(0.37)	(0.04)	(0.60)
7	81.15	85.70	84.49	94.16	85.46	86.34	87.13	89.54	88.40	88.13	96.15	88.33	88.20	92.70
	(1.28)	(0.43)	(5.76)	(1.33)	(3.08)	(0.52)	(2.97)	(0.57)	(0.78)	(1.87)	(0.30)	(0.38)	(1.45)	(0.22)
14	83.55	87.08	84.88	95.89	81.91	86.27	90.08	87.44	87.27	85.13	96.61	89.63	89.19	91.41
	(4.93)	(0.61)	(2.72)	(0.46)	(1.19)	(0.24)	(3.44)	(0.85)	(0.27)	(2.18)	(1.28)	(1.19)	(0.95)	(0.03)
Total Mean	83.57	85.12	84.59	94.82	85.07	86.05	89.20	87.37	87.95	86.14	95.75	89.43	88.67	92.34
	(1.42)	(0.55)	(1.84)	(0.40)	(1.14)	(0.24)	(1.39)	(1.07)	(0.26)	(1.25)	(0.39)	(0.48)	(0.40)	(0.40)

*MTC denotes microtracer F-Ni as marker.

*AIA (mtc) denotes acid insoluble ash as a marker from microtracer containing diet and fecal samples.

*AIA (Cr) denotes acid insoluble ash as a marker from Cr_2O_3 containing diet and fecal samples.

*AIA denotes acid insoluble ash as a marker from both Cr_2O_3 and microtracer containing diet and fecal samples.

*D denotes marker included / resulted from diet with two external marker.

*S denotes marker included / resulted from diet with only one external marker.

Table 3.3.3.14 : Analysis of variance for the ADC (%) of protein estimated on feces collected from fecal collection chamber at 6 and 24 hours after feeding over four different days of fecal collection using seven resulting markers.

Sources of variation	DF	SS	MS	F	P
Day (A)	3	11.3012	3.7671	0.5624	0.6420
Marker (B)	6	1228.98	204.83	30.5821	0.0000*
Collection Strategy (C)	1	212.879	212.879	31.7838	0.0000*
A x B	18	72.7998	4.0444	0.6039	0.8814
A x C	3	11.6807	3.8936	0.5813	0.6297
B x C	6	34.7523	5.7921	0.8648	0.5265
A x B x C	18	71.1214	3.9512	0.5899	0.8920
Error	56	375.07	6.6977		
Total	111	2018.59			

Table 3.3.3.12(b) : Multiple comparison of means for the apparent digestibility coefficients of protein estimated by using seven resulting markers in Trial 2 (ANOVA Table 3.3.3.14).

Markers	Mean ADC (%) of Protein
AIA (from diet with single Cr_2O_3 marker inclusion)	95.29 a1
AIA (from both marker included in same diet)	90.80 b
Cr_2O_3 (from both marker included in same diet)	87.36 c
Microtracer F-Ni (from both marker included diet)	87.25 cd
Cr_2O_3 (from diet with single Cr_2O_3 marker inclu.)	86.53 cd
Microtracer F-Ni (from diet with single microtracer marker inclusion)	85.47 cd
AIA (from diet with single microtracer inclusion)	85.36 d
Pooled Standard Error of Mean (n = 16) =	0.69.

[1]Mean values of components with the same superscript are not significantly different (P > 0.05).

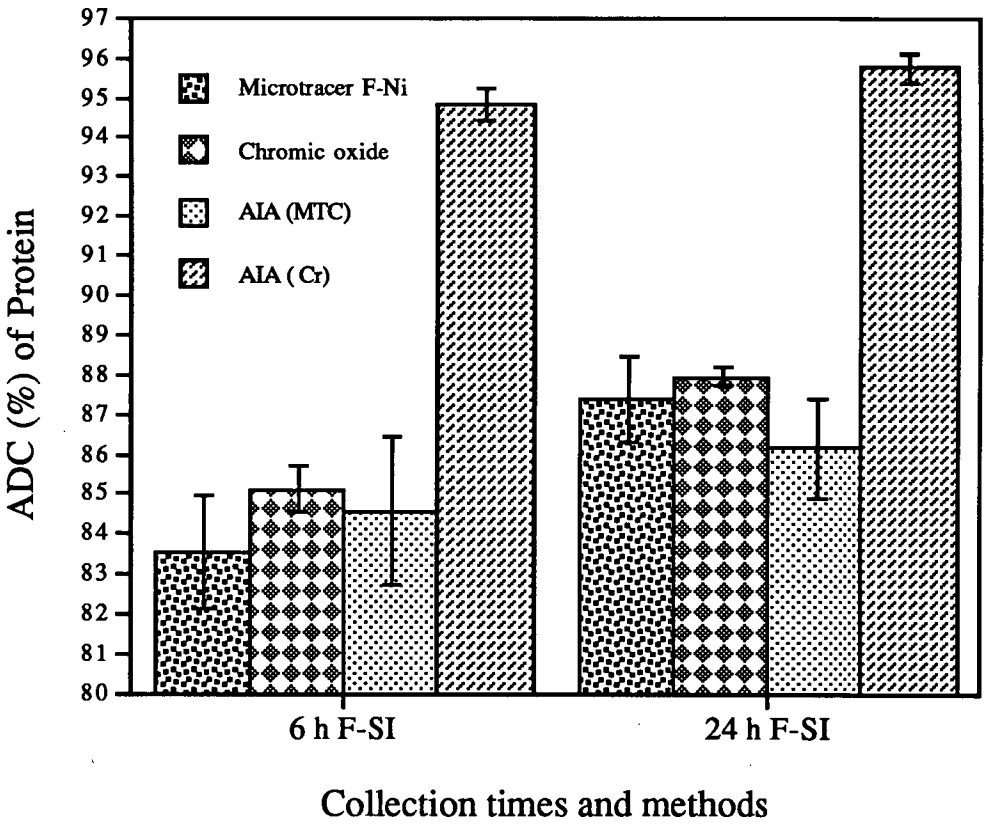


Figure 3.3.3.4 : The overall apparent digestibility coefficients (%) of protein (mean ± SE, n=8, considering days as replication) estimated on feces collected from fecal collection chamber at 6 and 24 hours after feeding over four different days of collection using four different markers with single inclusion.

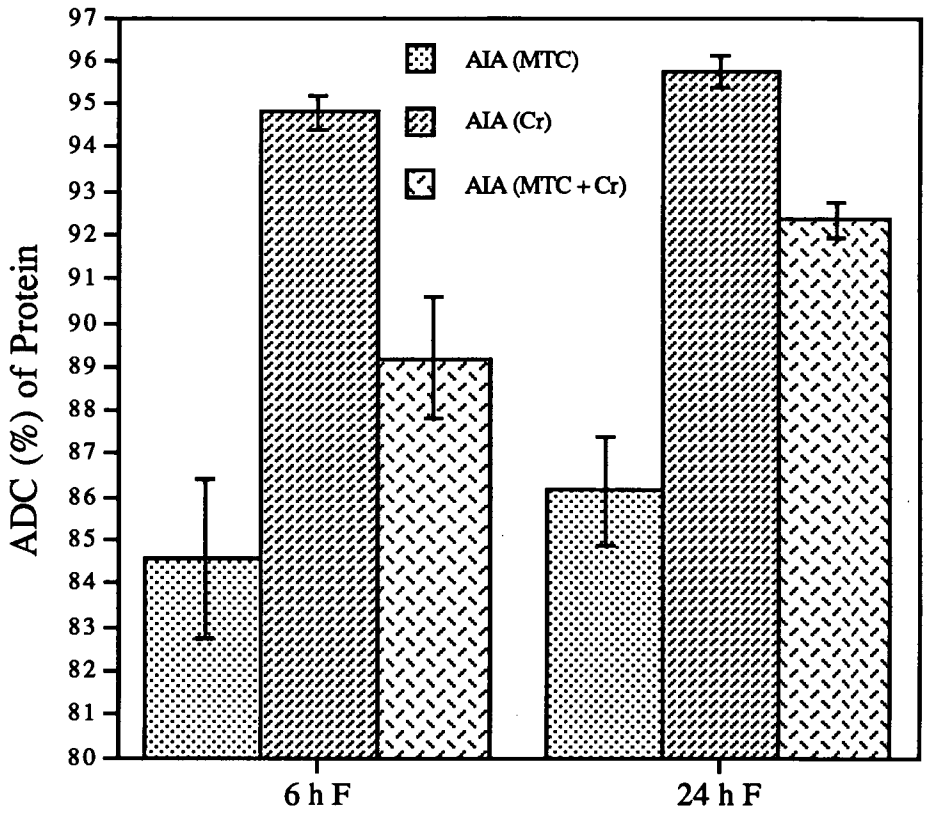


Figure 3.3.3.5 : The apparent digestibility coefficients (%) of protein (mean ± SE, n = 8, considering days as replication) estimated on feces collected from fecal collection chamber at 6 and 24 hours and using three sources of acid insoluble ash as markers.

3.3.3.3 Apparent digestibility coefficients of dry matter

Trial 1

The apparent digestibility coefficients (%) of dry matter in diets estimated using feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of collection over four different days in the experiment with microtracer F-Ni and chromic oxide as markers is shown in Table 3.3.3.15 and the analysis of variance for the same is shown in Table 3.3.3.16. The overall ADC (%) of dry matter using the above two markers over two collection methods and two different times of fecal collection is shown in Figure 3.3.3.6. Fecal collection days, time of collection and the use of markers resulted no significant effect on ADC of dry matter values ($P > 0.05$). Methods of fecal collection ie., collection from fecal chamber and by stripping exerted highly significant ($P < 0.001$) effect on dry matter digestibility values. The ADC of dry matter values estimated with feces collected from fecal chamber were higher than that of stripped samples. But stripped fecal samples at 6 and 24 hours collection resulted no significant ($P > 0.05$) difference on ADC values. Although the time of fecal collection was not significant but the interaction between collection time and collection method were significant ($P < 0.05$). None of the other interactions between the factors were significant ($P > 0.05$).

The apparent digestibility coefficients (%) of dry matter in diets estimated on feces collected from fecal collection chamber at 6 and 24 h interval of collection over four different days using

microtracer F-Ni, chromic oxide and two sources of acid insoluble ash as markers is shown in Table 3.3.3.17 and the analysis of variance for the same in Table 3.3.3.18. The overall ADC (%) of dry matter using the above four markers over two different times of fecal collection from fecal collection chamber is shown in Figure 3.3.3.7. Fecal collection days resulted no significant effect on ADC of dry matter values ($P > 0.05$). The markers used differed significantly ($P < 0.0001$) for the ADC of dry matter values. The ADC of dry matter values with acid insoluble ash markers remained significantly higher ($P < 0.05$) than that of microtracer F-Ni and chromic oxide markers. Even ADC of dry matter estimated using two sources of acid insoluble ash differed significantly ($P< 0.05$) amongst themselves. The ADC using AIA from microtracer containing diet were higher than using same from chromic oxide containing diet. Another interesting result with AIA markers were that the 6 h and 24 h fecal collection method had the same ($P>0.05$) ADC values.

Time of fecal collection ie., 6 h and 24 h after feeding also appeared to be a significant factor ($P<0.05$). None of the interactions between the factors were significant ($P>0.05$). The multiple comparison of means for the ADC of dry matter in diets estimated using four different markers with two methods of fecal collection and two different times of collection is shown in Table 3.3.3.19.

Table 3.3.3.15 : The apparent digestibility coefficients (%) of dry matter (mean with SE in parentheses, n = 2) in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding over four different days of fecal collection using microtracer F-Ni and chromic oxide as markers in Trial 1.

Days	Microtracer F-Ni marker				Chromic oxide marker			
	6 hours collection		24 hours collection		6 hours collection		24 hours collection	
	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber	Stripping	Fecal chamber
3	62.63	60.59	57.15	74.67	53.21	65.26	57.48	72.46
	(4.82)	(11.64)	(9.93)	(0.55)	(3.02)	(0.63)	(4.84)	(1.44)
5	54.32	64.69	51.60	75.00	64.84	63.28	59.45	70.60
	(14.85)	(4.15)	(5.82)	(0.42)	(4.96)	(7.05)	(1.52)	(1.44)
7	58.08	67.26	63.76	73.86	50.19	64.63	59.07	69.42
	(2.38)	(7.74)	(0.77)	(3.86)	(0.57)	(0.29)	(1.54)	(1.88)
14	66.78	63.04	53.59	65.37	67.25	67.84	60.06	73.08
	(5.66)	(1.70)	(7.60)	(0.68)	(1.24)	(0.41)	(1.13)	(0.42)
Total mean	60.44	63.89	56.52	72.22	58.87	65.25	59.01	71.39
	(4.20)	(3.26)	(3.14)	(1.94)	(2.98)	(1.48)	(1.09)	(0.76)

Table 3.3.3.16 : Analysis of variance for the apparent digestibility coefficients (%) of dry matter (with arc sin square root transformation) in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding over four different days using microtracer F-Ni and chromic oxide as indicators.

Sources of variation	DF	SS	MS	F	P
Day (A)	3	0.00291	0.00097	0.1760	0.9119
Marker (B)	1	0.00012	0.00012	0.0213	0.8850
Time (C)	1	0.0131	0.0131	2.3808	0.1327
Collection Method (D)	1	0.15719	0.15719	28.557	0.0000*
A x B	3	0.02606	0.00869	1.5783	0.2138
A x C	3	0.02339	0.00780	1.4162	0.2561
A x D	3	0.00991	0.0033	0.5999	0.6198
B x C	1	0.00032	0.00032	0.0577	0.8117
B x D	1	0.00003	0.00003	0.0054	0.9418
C x D	1	0.03800	0.03800	6.9045	0.0131*
A x B x C	3	0.00313	0.00104	0.1894	0.9028
A x B x D	3	0.02026	0.00675	1.2271	0.3159
A x C x D	3	0.01655	0.00551	1.0025	0.4044
B x C x D	1	0.00408	0.00408	0.7418	0.3955
A x B x C x D	3	0.00387	0.00129	0.2346	0.8716
Error	32	0.17614	0.0055		
Total	63	0.4951			

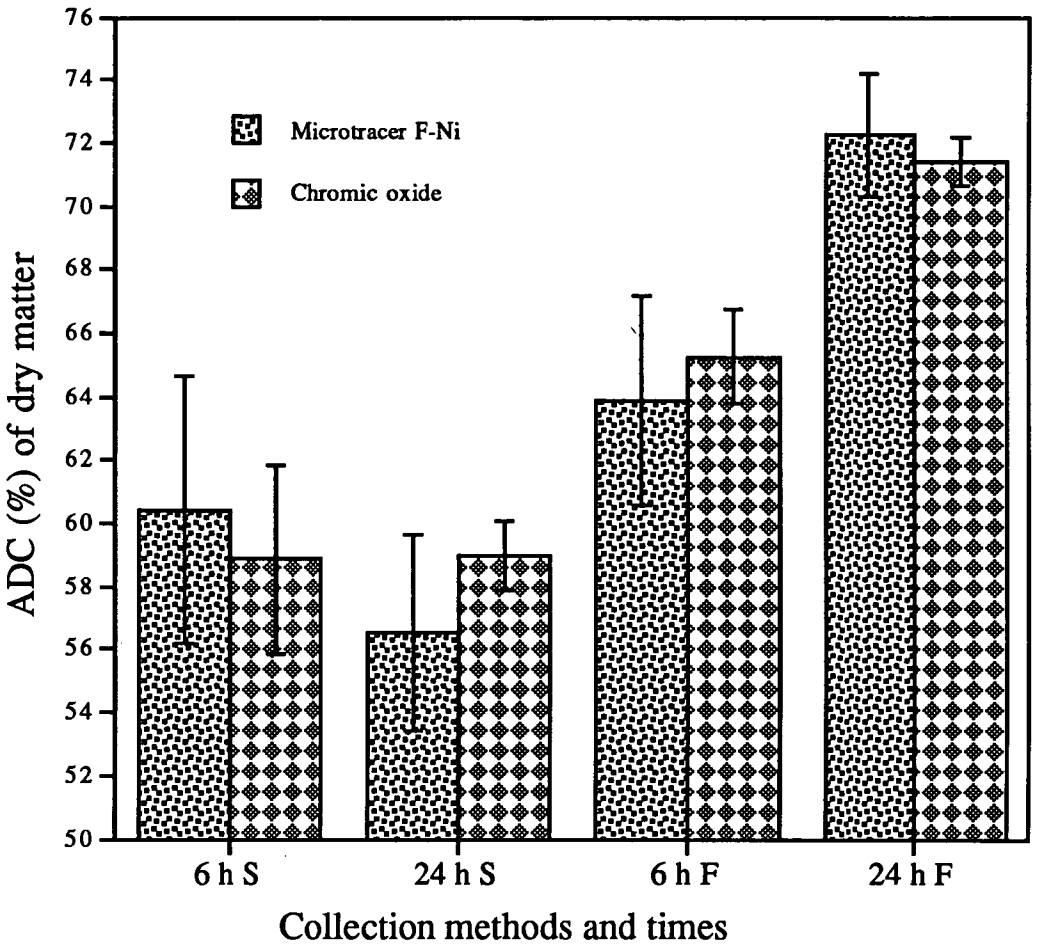


Figure 3.3.3.6 : The overall apparent digestibility coefficients of dry matter (mean ± SE, n = 8 considering days as replication) in diets estimated on feces collected from fecal collection chamber (F) and by stripping (S) at 6 and 24 hours interval of feeding using microtracer and chromic oxide as markers (in Trial 1).

Table 3.3.3.17 : The apparent digestibility coefficients (%) of dry matter (mean with SE in parentheses, n = 2) in diets estimated on feces collected from fecal collection chamber at 6 and 24 hours interval of feeding over four different days of fecal collection using microtracer F-Ni, chromic oxide and acid insoluble ash (AIA) as markers in Trial 1.

Days	Microtracer F-Ni marker		Chromic oxide marker		AIA marker (from microtracer - diet)		AIA marker (from Cr_2O_3 - diet)	
	6 hours collection	24 hours collection	6 hours collection	24 hours collection	6 hours collection	24 hours collection	6 hours collection	24 hours collection
3	60.59	74.67	65.26	72.46	73.10	68.08	85.38	89.45
	(11.64)	(0.55)	(0.63)	(1.44)	(10.90)	(3.24)	(0.84)	(0.68)
5	64.69	75.00	63.28	70.60	76.91	77.82	84.65	85.79
	(4.15)	(0.42)	(7.05)	(1.44)	(6.12)	(1.73)	(3.62)	(1.13)
7	67.26	73.86	64.63	69.42	75.51	77.67	87.18	85.94
	(7.74)	(3.86)	(0.29)	(1.88)	(7.58)	(-)	(2.06)	(2.96)
14	63.04	65.37	67.84	73.08	79.00	74.00	87.14	84.19
	(1.70)	(0.68)	(0.41)	(0.42)	(2.49)	(5.50)	(2.27)	(4.44)
Total mean	63.89	72.22	65.25	71.39	76.13	73.92	86.09	86.34
	(3.26)	(1.94)	(1.48)	(0.76)	(2.92)	(5.78)	(1.00)	(1.27)

Table 3.3.3.18 : Analysis of variance for the apparent digestibility coefficients (%) of dry matter in diets estimated on feces collected from fecal collection chamber at 6 and 24 hours interval of feeding over four different days using microtracer F-Ni, chromic oxide and two sources of acid insoluble ash as indicators.

Sources of variation	DF	SS	MS	F	P
Day	3	23.4836	7.8279	0.2056	0.8918
Marker	3	3478.50	1159.5	30.453	0.0000*
Time	1	172.069	172.069	4.5192	0.0413*
Day x Marker	9	235.51	26.1678	0.6873	0.7148
Day x Time	3	66.215	22.0716	0.5797	0.6326
Marker x Time	3	267.136	89.045	2.3387	0.0921
Day x Marker x Time	9	83.4098	9.2633	0.2434	0.9848
Error	32	1218.39	38.075		
Total	63	5544.71			

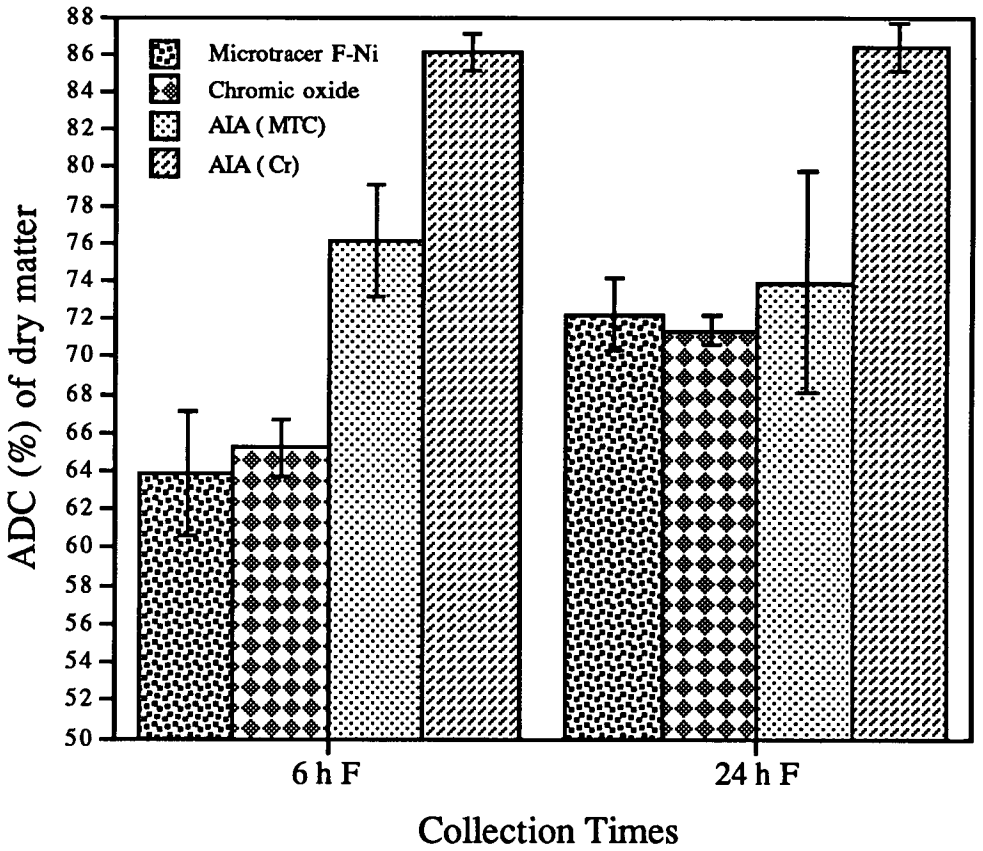


Figure 3.3.3.7 : The overall apparent dry matter digestibility coefficients (mean ± SE, n = 8, considering days as replication) in diets estimated on feces collected from fecal collection chamber (F) at 6 and 24 hours interval of feeding using four different markers (in Trial 1).

Table 3.3.3.19 : Multiple comparison of means for the apparent digestibility coefficients (%) of dry matter (mean with SE in the parentheses, n=8, considering days as replication) in diets estimated on feces collected from fecal collection chamber and by stripping at 6 and 24 hours interval of feeding by Cr_2O_3, microtracer F-Ni and acid insoluble ash (AIA) as markers (in Trial 1).

Markers	6 hours collection		24 hours collection	
	Stripping	Fecal chamber	Stripping	Fecal chamber
Microtracer F-Ni	$_{e}$ 60.44 ^{a} (4.20)	$_{e}$ 63.89 ^{ab} (3.26)	$_{e}$ 56.52 ^{a} (3.14)	$_{e}$ 72.22 ^{b} (1.94)
Chromic oxide	$_{e}$ 58.87 ^{a} (2.98)	$_{e}$ 65.25 ^{b} (1.48)	$_{e}$ 59.01 ^{a} (1.09)	$_{e}$ 71.39 ^{c} (0.76)
AIA (MTC)[1]	-	$_{f}$ 76.13 ^{a} (2.92)	-	$_{e}$ 73.92 ^{a} (2.18)
AIA (Cr_2O_3)[2]	-	$_{g}$ 86.09 ^{a} (1.00)	-	$_{f}$ 86.34 ^{a} (1.27)

[1]Acid insoluble ash as a marker from microtracer F-Ni containing diets and feces.

[2]Acid insoluble ash as a marker from Cr_2O_3 containing diets and feces.

$^{abc}/_{efg}$ Mean values of components with the same superscript in same row and same subscript in same column are not significantly different ($P > 0.05$).

Trial 2

The apparent digestibility coefficients (%) of dry matter in diets estimated on feces collected from fecal collection chamber at 6 and 24 hours interval and stripping at 24 hours interval over four different days of fecal collection, using diets with microtracer F-Ni, chromic oxide and both of them as markers is shown in Table 3.3.3.20 and the analysis of variance for the same is shown in Table 3.3.3.21. The overall ADC (%) of dry matter using the above two markers with two inclusion levels (single or both), using three collection times and methods (6 h fecal collection, 24 h fecal collection and 24 h stripping) is shown in Figure 3.3.3.8 and the multiple comparison of means for the same in Table 3.3.3.24(a). Days of fecal collection and the use of markers resulted no significant effect ($P > 0.05$) on ADC of dry matter values. Inclusion of these two markers as single or both into experimental diets, as a whole, resulted a significant ($P > 0.05$) increase in ADC values for the diets incorporated with both markers. Time and method of fecal collection were highly significant ($P<0.001$) with 24 h fecal collection exerted the highest ADC values. The ADC values for 24 h stripping were similar ($P > 0.05$) to those for 6 h fecal collection.

Within the same time and collection method, inclusion level didn't affect ($P>0.05$) the ADC of dry matter values in case of microtracer marker, but the values with both marker remained higher than single marker inclusion in all collection situations. With chromic oxide marker ADC values at 6 h single inclusion differed ($P<0.05$) with the same for both inclusion. None of the interactions between the treatments were significant ($P>0.05$).

The apparent digestibility coefficients of dry matter estimated on feces collected from fecal collection chamber at 6 h and 24 h collection intervals over four different days of fecal collection using seven resulted markers is shown in Table 3.3.3.22 and the analysis of variance and multiple comparison of means for the same in Tables 3.3.3.23 and 3.3.3.24(b). The overall ADC of dry matter using the four markers with two different times of fecal collection at single inclusion level is shown in Figure 3.3.3.9. The ADC of dry matter using three sources of acid insoluble ash as markers is shown in Figure 3.3.3.10. The day of fecal collection were not significant ($P > 0.05$) at all. But the use of different markers resulted a highly significant ($P < 0.0001$) effect on ADC values. Among the different markers, microtracer F-Ni and chromic oxide were not significantly different from each other ($P>0.05$) for the ADC of dry matter values. But the ADC of dry matter values using acid insoluble ash (from chromic oxide and both marker containing diets) as markers remained significantly higher ($P<0.05$) with the highest ADC value for chromic oxide containing diets. Collection time was also highly significant ($P<0.0001$).

The trends of apparent digestibility coefficients of dry matter with microtracer F-Ni and chromic oxide as markers was found similar ($P >0.05$) for 6 h and 24 h fecal collections with all the inclusions. Fecal collection at 24 hour interval resulted the highest ADC values and were significantly different with 6 h fecal collection ($P<0.05$) for the diets containing microtracer F-Ni and chromic oxide . Acid insoluble ash (from both microtracer and chromic oxide containing diets) resulted similar ($P>0.05$) ADC of dry matter values for 6h and 24 h collections. ADC of dry matter with chromic oxide marker

(single inclusion) at 6 h fecal collection differed significantly ($P < 0.05$) with that of double included marker.

Table 3.3.3.20 : The apparent digestibility coefficients (%) of dry matter (mean with SE in parentheses, n = 2) estimated on feces collected from fecal collection chamber at 6 and 24 hours and by stripping at 24 hours after feeding over four different days of fecal collection using diets with microtracer F-Ni (D -1), Cr_2O_3 (D - 2) and both of them (D - 3) as markers in Trial 2.

Day	6 h collection from fecal chamber				24 h collection from fecal chamber				24 h collection by stripping			
	D-1	D-2	D-3		D-1	D-2	D-3		D-1	D-2	D-3	
	MTC[1]	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3	MTC	Cr_2O_3
3	52.82	51.48	55.77	55.86	58.81	66.57	66.91	62.07	51.71	58.38	59.59	57.66
	(6.29)	(0.19)	(0.32)	(3.88)	(8.55)	(0.10)	(3.26)	(3.58)	(5.39)	(1.32)	(0.65)	(1.15)
5	55.03	49.69	61.14	56.81	62.20	62.41	69.41	66.31	56.49	58.56	58.92	61.67
	(6.97)	(3.83)	(1.60)	(1.87)	(2.31)	(0.50)	(0.37)	(0.88)	(1.37)	(0.69)	(0.96)	(2.41)
7	46.78	56.82	57.06	59.70	65.44	64.37	64.49	64.30	49.44	55.85	57.71	57.31
	(0.65)	(2.17)	(8.63)	(2.14)	(3.13)	(2.60)	(1.20)	(2.08)	(1.41)	(1.05)	(1.23)	(1.50)
14	50.83	59.54	48.03	60.46	62.49	58.27	67.73	66.58	48.72	52.47	54.15	58.01
	(9.81)	(1.32)	(1.93)	(1.78)	(1.16)	(0.08)	(4.66)	(1.93)	(14.13)	(3.92)	(8.01)	(0.49)
Total	51.36	54.38	55.55	58.21	62.24	62.90	67.13	64.81	51.59	56.31	57.59	58.66
Mean	(3.25)	(1.73)	(2.83)	(1.21)	(2.31)	(1.26)	(1.49)	(1.12)	(3.58)	(1.24)	(2.01)	(0.88)

[1]MTC denotes microtracer F-Ni as a marker.

Table 3.3.3.21 : Analysis of variance for the ADC (%) of dry matter (transformed data) estimated on feces collected from fecal collection chamber at 6 and 24 h and by stripping at 24 hours after feeding over four different days of collection using microtracer F-Ni and chromic oxide as markers with single and both inclusion in experimental diets (Trial 2).

Sources of Variation	DF	SS	MS	F	P
Day (A)	3	0.0087	0.0029	0.8542	0.4713
Marker (B)	1	0.0063	0.0063	1.8533	0.1798
Inclusion (C) (single vs both)	1	0.0370	0.0370	10.8496	0.0019*
Collection Procedure (D)	2	0.1766	0.0883	25.8823	0.0000*
A x B	3	0.0095	0.0032	0.9250	0.4359
A x C	3	0.0014	0.0005	0.1320	0.9405
A x D	6	0.0077	0.0013	0.3740	0.8919
B x C	1	0.0034	0.0034	0.9936	0.3239
B x D	2	0.0077	0.0039	1.1244	0.3333
C x D	2	0.0002	0.0001	0.0275	0.9729
A x B x C	3	0.0072	0.0024	0.7019	0.5555
A x B X D	6	0.0217	0.0036	1.0591	0.4000
A x C x D	6	0.0148	0.0024	0.7247	0.6318
B x C x D	2	0.0013	0.00065	0.1916	0.8263
A x B x C x D	6	0.0074	0.0012	0.3629	0.8987
Error	48	0.1637	0.0034		
Total	95	0.4745			

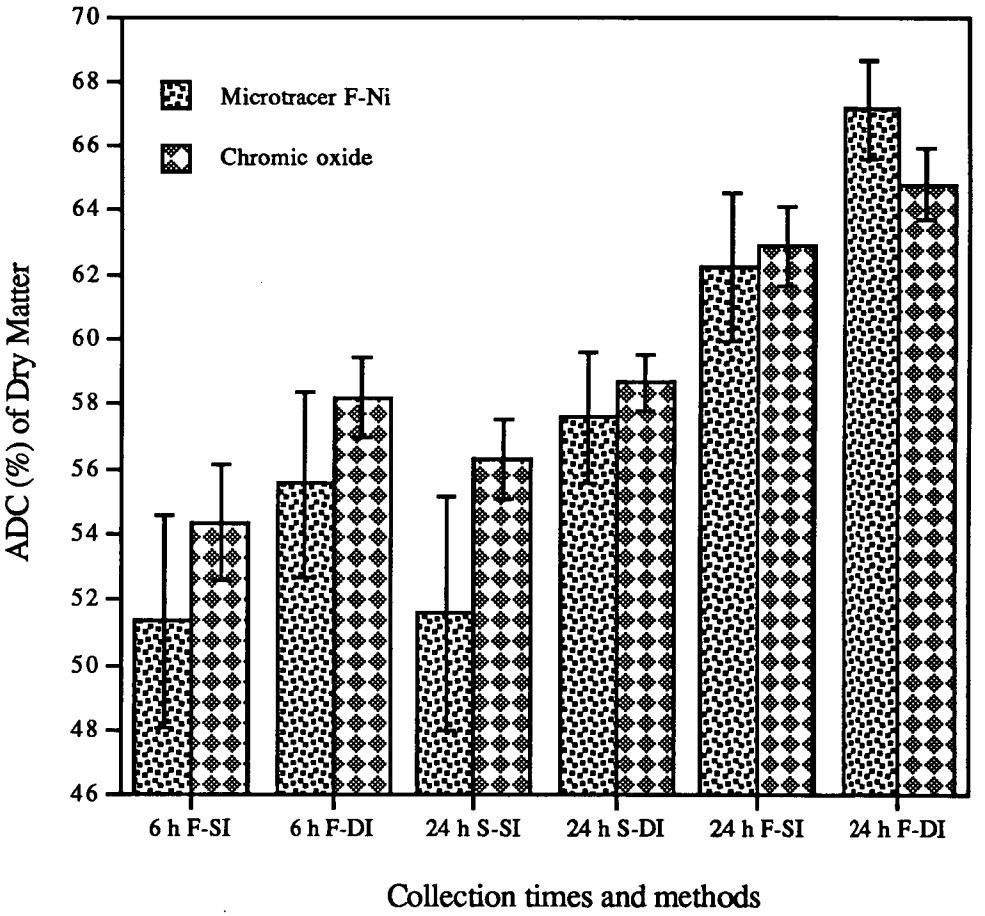


Figure 3.3.3.8 : The overall ADC (%) of dry matter (mean ± SE, n = 8, considering days as replication) in diets estimated on feces collected from fecal collection chamber at 6 hours (6 h F) and 24 hours (24 h F) and by stripping at 24 hours (25 h S) after feeding using microtracer and chromic oxide with two inclusion levels (single inclusion - SI, double inclusion - DI) as markers in Trial 2.

Table 3.3.3.22 : The apparent digestibility coefficients (%) of dry matter (mean with SE in parentheses, n = 2) estimated on feces collected from fecal collection chamber at 6 and 24 hours after feeding over four different days of collection and using seven markers.

Day	6 h collection from fecal chamber							24 h collection from fecal chamber						
	MTC (S)	Cr_2O_3 (S)	AIA (mtc-S)	AIA (Cr-S)	MTC (D)	Cr_2O_3 (D)	AIA (D)	MTC (S)	Cr_2O_3 (S)	AIA (mtc-S)	AIA (Cr-S)	MTC (D)	Cr_2O_3 (D)	AIA (D)
3	52.81	51.48	48.08	83.35	55.77	55.86	67.70	58.81	66.57	48.70	85.52	66.91	62.07	73.80
	(6.29)	(0.19)	(15.1)	(1.72)	(0.32)	(3.88)	(10.3)	(8.55)	(0.10)	(0.08)	(1.40)	(3.26)	(3.58)	(4.64)
5	55.03	49.69	58.10	83.86	61.14	56.81	70.02	62.20	62.41	67.83	85.57	69.41	66.31	79.34
	(6.97)	(3.83)	(1.64)	(1.30	(1.60)	(1.87)	(0.24)	(2.31)	(0.50)	(0.36)	(1.63)	(0.37)	(0.88)	(2.20)
7	46.78	56.82	57.29	86.92	57.06	59.70	62.18	65.44	64.37	60.63	88.17	64.49	64.30	77.68
	(0.65)	(2.17)	(12.8)	(0.24)	(8.63)	(2.14)	(8.15)	(3.13)	(2.60)	(7.57)	(1.00)	(1.20)	(2.08)	(2.13)
14	50.83	59.54	54.18	87.15	48.03	60.46	71.72	62.49	58.27	55.73	88.96	67.73	66.58	73.39
	(9.81)	(1.32)	(3.40)	(1.26)	(1.93)	(1.78)	(9.09)	(1.16)	(0.08)	(4.89)	(3.97)	(4.66)	(1.93)	(0.70)
Total Mean	51.36	54.38	54.41	85.32	55.55	58.21	67.90	62.24	62.90	58.22	87.05	67.13	64.81	76.05
	(3.25)	(1.73)	(4.73)	(0.81)	(2.83)	(1.21)	(3.83)	(2.31)	(1.26)	(3.63)	(1.05)	(1.49)	(1.12)	(1.65)

*MTC denotes microtracer F-Ni as marker.

*AIA (mtc) denotes acid insoluble ash as a marker from microtracer containing diet and fecal samples.

*AIA (Cr) denotes acid insoluble ash as a marker from Cr2O3 containing diet and fecal samples.

*AIA denotes acid insoluble ash as a marker from both Cr_2O_3 and microtracer containing diets.

*D denotes marker included / resulted from diet with two external markers.

*S denotes marker included / resulted from diet with only one external marker.

Table 3.3.3.23 : Analysis of variance for the ADC (%) of dry matter estimated on feces collected from fecal collection chamber at 6 and 24 hours and by stripping at 24 hours after feeding over four different days of collection using seven resulting markers (Trial 2).

Sources of variation	DF	SS	MS	F	P
Day (A)	3	188.85	62.95	1.3878	0.2560
Marker (B)	6	11289.8	1881.6	41.4826	0.0000*
Collection Strategy (C)	1	1505.29	1505.29	33.1858	0.0000*
A x B	18	586.44	32.5798	0.7183	0.7782
A x C	3	31.002	10.334	0.2278	0.8766
B x C	6	309.738	51.623	1.1381	0.3526
A x B x C	18	485.302	26.96	0.5944	0.8886
Error	56	2540.12	45.359		
Total	111	16936.5			

Table 3.3.3.24 (a): Multiple comparison of means for the apparent digestibility coefficients (%) of dry matter (mean with SE in the parentheses, n = 8, considering days as replication) in diets estimated on feces collected at 6 and 24 hours after feeding using microtracer F-Ni and chromic oxide (included as single or both into diets) as markers in Trial 2.

Markers	6 hours fecal collection		24 hours fecal collection		24 hours stripping	
	Single inclusion	Double inclusion	Single inclusion	Double inclusion	Single inclusion	Double inclusion
Microtracer F-Ni	$_{e}$ 51.36 ^{a} (3.25)	$_{e}$ 55.55 ^{a} (2.83)	$_{e}$ 62.24 ^{bc} (2.31)	$_{e}$ 67.13 ^{c} (1.49)	$_{e}$ 51.59 ^{a} (3.58)	$_{e}$ 57.59 ^{ab} (2.01)
Chromic oxide	$_{e}$ 54.38 ^{a} (1.73)	$_{e}$ 58.21 ^{b} (1.21)	$_{e}$ 62.90 ^{c} (1.26)	$_{e}$ 64.81 ^{c} (1.12)	$_{e}$ 56.31 ^{ab} (1.24)	$_{e}$ 58.66 ^{b} (0.88)

$^{abc}/_{e}$ Mean values of components with the same superscript in same row and same subscript in same column are not significantly different ($P > 0.05$).

Table 3.3.3.24(b) : Multiple comparison of means for the apparent digestibility coefficients of dry matter estimated by using seven resulting markers in Trial 2 (ANOVA Table 3.3.3.23).

Markers	ADC (%) of Dry Matter
AIA (from diet with single Cr_2O_3 marker inclusion)	86.19 a1
AIA (from both marker included in same diet)	71.98 b
Cr_2O_3 (from both marker included in same diet)	61.51 c
Microtracer F-Ni (from both marker included diet)	61.32 cd
Cr_2O_3 (from diet with single Cr_2O_3 marker inclu.)	58.64 cd
Microtracer F-Ni (from diet with single microtracer marker inclusion)	56.80 cd
AIA (from diet with single microtracer inclusion)	56.32 d
Pooled Standard Error of Mean (n = 16) =	1.83

[1]Mean values of components with the same superscript are not significantly different (P > 0.05).

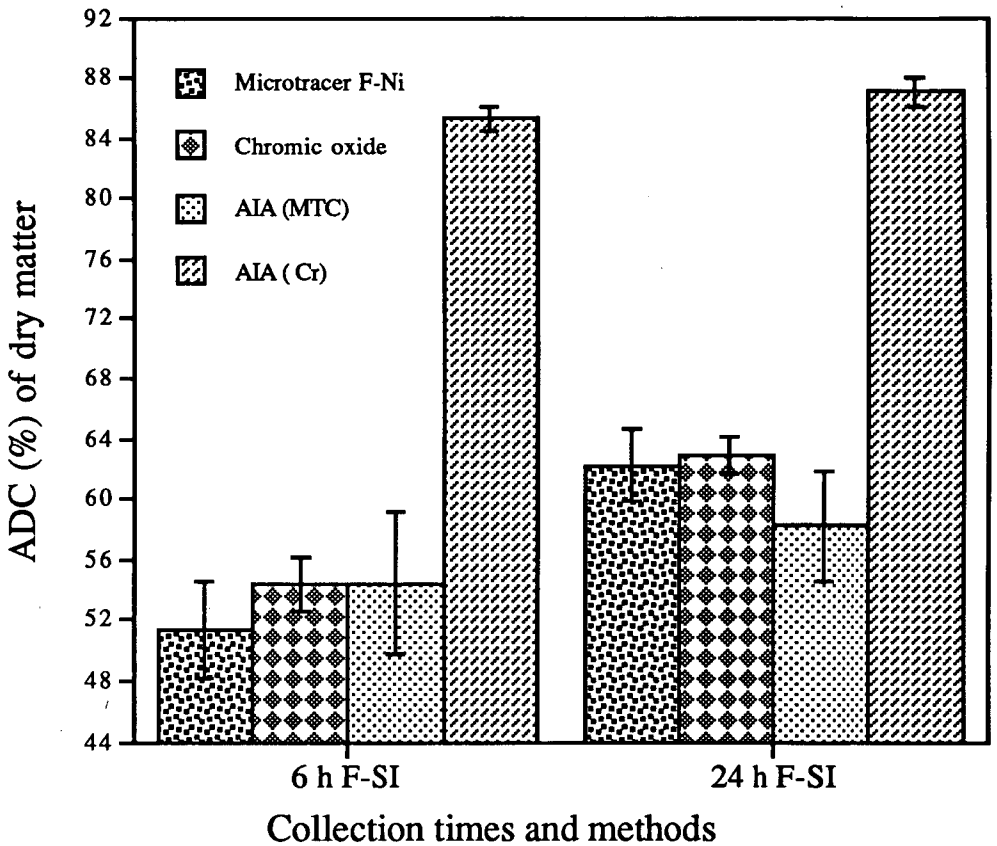


Figure 3.3.3.9 :The overall apparent digestibility coefficients (%) of dry matter (mean ± SE, n=8, considering days as replication) estimated on feces collected from fecal collection chamber at 6 and 24 hours after feeding over four different days of collection using four different markers with single inclusion (in Trial 2).

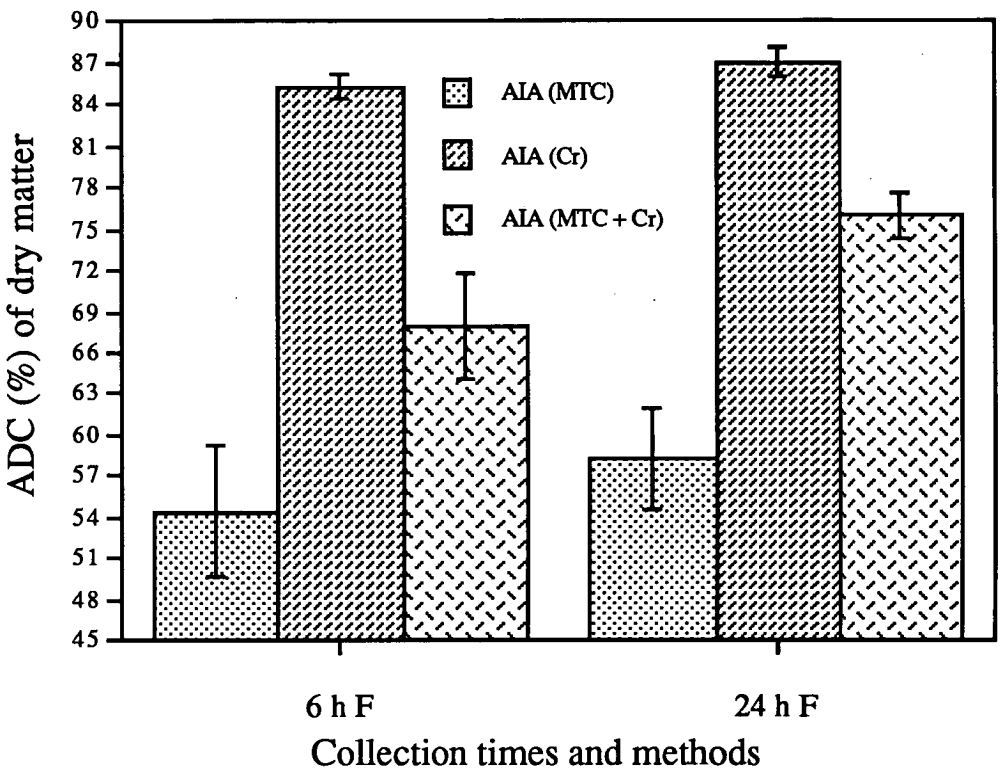


Figure 3.3.3.10 : The apparent digestibility coefficients (%) of dry matter (mean ± SE, n=8, considering days as replication) estimated on feces collected from fecal collection chamber at 6 and 24 hours and using three sources of acid insoluble ash as markers.

3.3.3.4 Fish growth performance

The effects of diets with three different combinations of marker on feed intake, specific growth rate and food conversion ratio as observed in Trial 2 is shown in Table 3.3.3.25. The feed intake were higher ($P<0.05$) with chromic oxide diet but there were no significant differences ($P>0.05$) between specific growth rate and food conversion ratios with these three experimental diets.

Table 3.3.3.25 : Effects of diets with three combination of external markers (1% Cr_2O_3, 1% microtracer F-Ni, and 1% Cr_2O_3 + 1% microtracer F-Ni) on feed intake, specific growth rate and food conversion ratio of rainbow trout, *O. mykiss* over a period of 25 days.

Components	Diet with 1% Cr_2O_3	Diet with 1% microtracer F-Ni	Diet with 2% marker (1% of each)
Initial weight (g)	96.61 ± 2.61	108.55 ± 3.01	104.65 ± 2.56
Final weight (g)	138.29 ± 3.28	151.07 ± 4.12	144.61 ± 3.17
Weight gain (g)	41.68 [a1] ± 4.67	42.52 [a] ± 1.75	39.96 [a] ± 3.27
% weight gain	43.04 [a] ± 4.26	39.38 [a] ± 2.36	38.29 [a] ± 3.49
Feed intake (% b.w per day)	0.40 [a] ± 0.01	0.32 [b] ± 0.01	0.35 [b] ± 0.003
SGR (% / day)	1.42 [a] ± 0.13	1.33 [a] ± 0.07	1.29 [a] ± 0.10
FCR	0.97 [a] ± 0.12	0.85 [a] ± 0.03	0.92 [a] ± 0.08

1Within each row, values with a common superscript are not significantly different ($P > 0.05$).

3.3.4 Discussion

Trial 1

On the basis of apparent digestibility coefficients of protein and dry matter (Tables 3.3.3.9, and 3.3.3.19) the most reliable and consistent estimates were obtained when using chromic oxide and microtracer F-Ni as dietary markers. The performance of acid insoluble ash as a dietary marker in this experiment was disappointing. The ADC values for both protein and dry matter were significantly ($P<0.05$) higher when using acid insoluble ash as marker than those of either chromic oxide or microtracer F-Ni markers. Even using acid insoluble ash from chromic oxide containing samples resulted significantly higher ($P<0.05$) ADC values than using acid insoluble ash from microtracer containing samples. These results are contradictory to the studies of Tacon and Rodrigues (1984) with rainbow trout, where acid insoluble ash was found to give significantly lower ($P<0.05$) digestibility coefficients than either chromic oxide or crude fibre. Getachew (1988) found with *Oreochromis niloticus* consistently lower ($P<0.05$) digestibility coefficients when hydrolysis resistant ash (HRA) were used as marker when compared to results obtained with other markers such as ash and hydrolysis resistant organic matter. The higher ADC values using acid insoluble ash in this experiment were in agreement with the studies of De Silva and Perera (1983) with the Asian cichlid *Etroplus suratensis* where hydrolysis resistant ash (HRA) was found to give consistently higher digestibility coefficients than either crude fibre or hydrolysis resistant organic matter as dietary markers.

Bowen (1981) also estimated higher digestibility values when HRA was used as a reference marker than HROM marker.

The ash and acid insoluble ash content in the diet and feces related with chromic oxide were very low in comparison with microtracer containing samples (Tables 3.3.3.1 to 3.3.3.4). The higher acid insoluble ash content from microtracer containing samples than chromic oxide containing samples may be explained by the loss of chromic oxide during ashing at 550°C for acid insoluble ash determination (Katz, et al., 1981). On the other hand, in a supplementary experiment, it was found that there was no loss of microtracer F-Ni during ashing or acid treated ashing. The loss of chromic oxide during ashing might result in low determination of acid insoluble ash which subsequently affect the digestibility values. The digestibility coefficients of protein and dry matter increased with increased duration of collection intervals (Figures 3.3.3.1, 3.3.3.2, 3.3.3.6 and 3.3.3.7). This was true for ADC values obtained with microtracer F-Ni and chromic oxide markers. Within each collection interval, the feces from fecal collection chamber resulted in higher digestibility values than feces collected by stripping for both chromic oxide and microtracer F-Ni markers. The ADC of protein and dry matter determined with feces collected by stripping at 6 and 24 hours after feeding were not significantly different ($P > 0.05$). The digestibility coefficients for 6 h fecal collection with microtracer F-Ni containing samples mirrored the results with stripping. However, this observation was not apparent with chromic oxide containing samples. The ADC of protein and dry matter determined with acid insoluble ash (two sources) for 6 h and 24 h fecal collection from fecal chamber were similar ($P > 0.05$).

The higher digestibility coefficient values with increased collection interval as observed in this experiment were in agreement with Windell et al. (1978), Vens-Cappel (1985) and Hajen et al. (1993). Windell et al. (1978) determined the effect of the time the feces remain in water before they are collected on the loss of nutrients and found that the major loss was incurred during the first hour of immersion. During this time about 21% of the dry matter, 12% of the protein and 4% of the lipids were lost increasing the digestibility coefficients by 11.5, 10 and 3.7% respectively. Within 16 hours the losses of nutrients from the feces reached 31, 12 and 9.8% respectively and the increase in digestibility coefficients were 17, 10 and 8.2%. Since all fecal nutrient losses are treated as if the nutrients had been absorbed by the fish, any leaching will result in erroneously high digestibility coefficients. Hajen et al. (1993) also reported that digestibility coefficients for protein increased by 12.4% units when calculations were based on fecal samples which had remained in water for a maximum of 18 hours. The results of Hajen et al.(1993) and Windell et al.(1978) were contrary to those of Cho and Slinger (1979) who found similar digestibility coefficients when feces were obtained by dissection and from fecal collection chambers after an overnight collection period of 15.5 hours. Similar results were also reported by Satoh et al. (1992) where they found collection of feces from collection chamber of 'Guelph system' and 'TUF column' after 15 hours of immersion had the same digestibility coefficients as 3 hours of immersion period. Cho et al. (1985) stressed that leaching is minimised when feces remain in undisturbed water and that most losses occur if the fecal pellets are broken up during the collection process. It was observed in this experiment that

the higher digestibility of protein and dry matter with 24 hours fecal collections (as compared with 6 h fecal collection) by 3.6 and 1.7 percents with microtracer F-Ni as a marker and 2.2 and 9.4 percents with chromic oxide marker. These respective differences are much lower than as reported by Hajen et al. (1993) although they estimated digestibility coefficients after 18 hours of immersion period. The fecal collection chamber developed in the present experiments was based on the 'Guelph system' as described by Cho and Slinger (1979). The smaller change in digestibility values with this experiment than Hajen et al. (1993) might be due to the different fecal collection systems. There were no information available about the changes in digestibility coefficients with 24 hrs of immersion using 'Guelph system' or any other systems. In this experiment, when the fecal collector tube was removed from the tank, there might have been a slight disturbance of the settled fecal mass as result of water gushing down from the tank, leading to some nutrient and dry matter loss. Considering the much lower percentage increase of digestibility coefficients with this modified 'Guelph system' at 24 h fecal collection, it might be concluded that the results of this experiment followed the findings of Cho and Slinger (1979).

The collection of feces by stripping always resulted in lower digestibility coefficients (Tables 3.3.3.5 and 3.3.3.15) than fecal collections from fecal chamber. These results were in agreement with Spyridakis et al. (1989), Hajen et al. (1993) amongst others. Spyridakis et al. (1989) studied the different methods of fecal collection and found that protein digestibility coefficients were low with stripping method than dissection, anal suction and much lower than filtration and decantation. Similar results were obtained by

Hajen et al. (1993) and Vens-Cappell (1985). These lower estimates might be due to the fact that the stripped feces contains residues of not yet digested (Gauthier and Landis, 1972 and Georgopoulou et al., 1985 confirmed the ability of enterocytes from the posterior intestine to ingest intact proteins by pinocytosis) or absorbed nutrients resulting an under estimation of digestibility or the stripped feces contains traces of urine, slime, serum, epithel of intestine and even sexual products all of which result in a decreased digestibility (Vens-Cappell, 1985). As the stripping method only sampled feces at one instant of time, another source of error may be due to non-uniformity of the digestibility process leading to diel variations in nutrient absorption (Hajen et al., 1993). The daily fluctuations in digestibility observed by other investigators (De Silva and Perera, 1984) suggests that stripping should not be considered as a representative method for digestibility estimations. However, Austreng (1978) recommends the fecal collection technique by stripping from hindmost part of the rectum ie. from ventral fin to anus of the fish.

Trial 2

In this trial the ADC values generated for protein and dry matter using microtracer and chromic oxide as markers followed the similar trends as in Trial 1 (Tables 3.3.3.12 and 3.3.3.24). Acid insoluble ash resulted inconsistent results (Figures 3.3.3.4, 3.3.3.5, 3.3.3.9 and 3.3.3.10).

Inclusion of markers (single vs both) into the diet resulted significant effect ($P < 0.05$) on ADC of protein and dry matter (Tables 3.3.3.11 and 3.3.3.21) with higher values for the diets with both markers. But

within each collection time and method the inclusion levels resulted no significant differences (P> 0.05) using microtracer F-Ni as a marker. At 6 hour fecal collection and using chromic oxide as a marker single inclusion varied significantly (P<0.05) with double inclusion for both ADC of protein and dry matter values. Fecal collection at 6 hours interval resulted similar ADC values with fecal collection by stripping at 24 hours.

Tacon and Rodrigues (1984) incorporated chromic oxide, polyethylene and acid washed sand into the same diet for trout and found that the ADC of nutrients generated by polyethylene and acid insoluble ash were always lower than determined by chromic oxide as marker. They compared the ADC values for different concentration levels of markers into the same diet. No reports were available to compare the results of this trial for the relationship between single and/or more inclusion of external markers into the similar diets. However, as no significant differences were found within each collection method and time using microtracer F-Ni and chromic oxide markers (except 6 h fecal collection using chromic oxide marker in double inclusion level), it might be concluded that both single and double inclusion of these two markers in diets with trout would be possible and comparable for digestibility studies.

Conclusion

In this experiment the digestibility coefficients determined with stripping or fecal chamber collection at 6 or 24 hours of interval using chromic oxide and microtracer F-Ni markers were in the line of general trends of digestibility estimations. The results of ADC values generated after incorporation of microtracer F-Ni and

chromic oxide into the same diet also confirmed the suitability of using microtracer F-Ni as a dietary marker for digestibility studies. It might be concluded, therefore, that microtracer F-Ni could be used as a successful dietary external marker for digestibility estimations with rainbow trout.

Chapter Three

Experiment Four

Estimation of apparent nutrient digestibility of ingredients using Microtracer F-Ni, Chromic oxide and Acid insoluble ash as markers in the same diet

3.4.1 Introduction

Measurement of digestibility gives a good indication of the availability of energy and nutrients in ingredients (Cho et al., 1985), thus providing a rational basis upon which diets can be formulated to meet specified standards of available nutrient levels (De Silva et al., 1990).

Though feeding of single ingredients to fish is often practised, it can be a very inefficient use of feed because a single ingredient is most unlikely to supply all the nutrients required by the animal in the balance in which it needs them (New, 1987). Feeding a single ingredient or test substances assumes that there are no interactions between classes affecting digestibility. This has been shown to be a false assumption by Watanabe (1977, as cited by Cho and Slinger, 1979) who found carbohydrate digestion to be influenced by level of fat in the diet. Other interactions are also known to exist (Cho and Slinger, 1979).

Very few potentially useful feed ingredients can be fed voluntarily as the sole component of a diet thus it is always necessary to combine a mixture of feed components in formulating diet. Thus determination of digestibility of a ingredient requires comparing the digestibilities of a reference and a test diet; the test diet being a mixture of the reference diet, test ingredient and a digestion indicator (Cho et al., 1985). The formula of reference diet and test diet combination, first introduced by Cho and Slinger (1979), are as follows :

	Reference diet (%)	Test diet (%)
Test ingredient	0	29.7
Basal diet	99.0	69.3
Marker, eg., Cr_2O_3	1.0	1.0

Inclusion of an inert indicator in the reference diet allows the digestibility coefficients of the nutrients in the test diets to be calculated from measurements of the nutrient to indicator ratios in the diet and feces. Once these coefficients have been calculated for the reference and test diets, the corresponding digestibility coefficients can be calculated for the nutrients in the ingredient being tested (Cho et al., 1985). The nutritive value of a nutrient in the test ingredient is designated as the digestion coefficient. A 100% digestion coefficient means that the fish can digest all of this particular nutrient provided by the ingredient in the diet (Law, 1984).

The use of reference diet assumes that there are no interaction between the components of the diet during digestion (Cho et al., 1985). As well, adoption of this procedure allows the preparation of an adequately balanced diet with which to test the susceptibility of the feedstuff to digestion. In determinations using reference and substituted diets, measurement of feed intake and growth rate allowed confirmation of the nutritional adequacy of the experimental diets.

The use of fish meal as an ingredient in test diet has been well established (Cho and Slinger, 1979). The replacement of fish meal by sunflower meal as a protein source in trout have also been studied

by Tacon et al. (1984), Jackson et al. (1982), amongst others. But the apparent digestibility coefficients of nutrients in these ingredients were determined by using Cr_2O_3 as an indicator. There are very few information on the use of more than one indicator in digestibility estimations of dietary ingredients. De Silva et al. (1990) used crude fibre and chromic oxide as indicators for the digestibility estimations of leaf meal (commercially available and commonly used in poultry feeds) in *Oreochromis aureus* and found that ingredient digestibilities estimated by using crude fibre as a marker were consistently higher than those estimated with chromic oxide. However, the use of crude fibre as dietary marker were criticised by several workers as it has been reported to be assimilated in a small extent by certain species (Van Dyke and Sutton, 1977). Chromic oxide were also reported to move in a differential rate in *Tilapia mossambica* (Bowen, 1978).

Considering the availability of little or no information and sometimes contrary information, this experiment aimed to :

1) estimate the apparent digestibility coefficients of dry matter and protein for fish meal and sunflower seed meal by rainbow trout using micro tracer F-Ni and chromic oxide as external indicators and acid insoluble ash as internal indicator in the same diet.

2) check the validity of using micro tracer F-Ni as an external indicator in ingredient digestibility estimations by comparing the values obtained by widely used chromic oxide and acid insoluble ash indicators.

3) estimate and compare the growth performance of rainbow trout by using fish meal and sunflower seed meal as ingredients.

3.4.2 Materials and Methods

3.4.2.1 Experimental System

Two separate experiments, each lasting 10 days, were carried out in 4 of the experimental tanks described in Chapter 2. Two tanks were selected for reference and test diets respectively. Water temperatures varied from 9.0 to 12.5°C for the first experiment when testing sunflower meal and 9.0 to 13.9°C for the second experiment with testing fish meal. The tanks were provided with constant aeration in natural day light / dark period conditions.

3.4.2.2 Fish

Rainbow trout (*O. mykiss*) were collected from National Key Centre for Aquaculture, University of Tasmania and distributed randomly between the tanks at a stocking density of 13 fish per tank. The initial and final weight of the fish were recorded before and after the experiment, which are presented in Table 3.4.3.9 and 3.4.3.10. The fish were acclimatised in the tanks for three days before starting experimental feeding. During that time they were fed commercial trout pellet (Gibson Feed Mill, Tasmania).

3.4.2.3 Diets

The composition of the basal diet is presented in Table 3.4.2.1. The basal diet were prepared by mixing the ground ingredients in a Atlas Food Mixer (model V-20) and blended for 20 minutes. The combination of basal diets, markers and test ingredients for the preparation of test and reference diets are presented in Table 3.4.2.2. All the compositions were determined on air-dry basis. The

reference diet and test diets for each experiment were prepared separately in two times and stored according to the procedure described in Experiment 2. The crude protein (N x 6.25), ash and marker contents in all the diets are presented in Table 3.4.2.3.

3.4.2.4 Feeding and fecal collection

The fish were fed *ad libitum* with experimental diets twice a day at 09.30 and 16.30 hours respectively. The amount of diet consumed per tank per day were recorded. The fecal collectors were cleaned with brush half an hour after feeding to remove the feed and fecal residues. Fecal collections were made in the morning (prior to the first feeding; ie., feces voided between 17.00 - 09.00 hours) from fecal collectors only. Collection of feces were started after 3rd day of feeding (De la Noüe et al., 1980) and were followed by 5th, 7th and 10th days after first feeding. All the samples dried in the oven at 80°C for 24 hours, ground and stored in a desiccator for subsequent analyses.

3.4.2.5 Analytical Procedures

The microtracer particles were recovered by using the magnetic wand, developed for counting as the methods described in Chapter 2, and the recovery (% w/w) were calculated.

Ash content were determined by placing the sample into a furnace at 550^{0}C for 6 hours. Using the ash residues, acid insoluble ash (AIA) were determined by treating the residues with acid and re-ashing at 550^{0}C for 2 hours (AOAC, 1980).

Nitrogen and crude protein content of the feed and fecal samples were determined and calculated according to the methods described in Experiment 3.

Chromium content of the experimental diets and feces were determined according to the methods described in Experiment 3.

3.4.2.6 Digestibility and fish performance calculations

The apparent digestibility coefficients (ADC) for protein were calculated by the following formula (Maynard and Loosli, 1969) :

$$\text{ADC (\%)} = 100 \left(1 - \frac{\text{\% marker in diet}}{\text{\% marker in feces}} \times \frac{\text{\% protein in feces}}{\text{\% protein in diets}}\right).$$

Apparent digestibility coefficients (ADC) for dry matter were calculated by the following formula (Barash et al., 1983) :

$$\text{ADC (\%)} = 100 \left(1 - \frac{\text{\% marker in diet}}{\text{\% marker in feces}}\right).$$

Apparent digestibility coefficients (ADC) for dry matter and protein for test ingredient were calculated by the following formula (Cho and Slinger, 1979) :

$$\text{ADC (\%)} = \frac{100}{30}\left(\text{ADC of test diet} - \frac{70}{100}\,\text{ADC of reference diet}\right).$$

Table 3.4.2.1. Formulation of basal diet used in this experiment.

Ingredients	Content (g / kg)
Fish meal (71.6% CP)	320.00
Wheat gluten flour	275.70
Soybean meal (defatted)	275.70
Vitamin premix [1]	2.00
Choline chloride	2.00
Mineral premix [2]	3.50
Fish oil	121.10

[1]The vitamin premixes supplied the following (mg / kg of dry diet unless otherwise indicated) : inositol, 400; niacin, 300; pantothenate (as D - calcium pentothenate), 166; menadione (as hetrazeen), 26.1; riboflavin, 60; pyridoxine (as pyridoxine-HCl), 36.5; thiamine (as thiamine mononitrate), 36.3; folic acid, 20; biotin, 3; vitamin B-12, 0.06; retinol acetate, 10000 IU; cholecalciferol, 2400 IU; di-α-tocopheryl acetate, 600 IU (Hajen et al., 1993b).

[2]The mineral premixes supplied the following amounts per kg of dry diet : magnesium (as $MgSo_4.7H_2O$), 250 mg; iron (as $FeSO_4.7H_2O$), 75 mg; zinc (as $Zn\ SO_4.7H_2O$), 60 mg; manganese (as $MnSO_4.H_2O$), 75 mg; copper (as $CuSO_4.5H_2O$), 6mg; florine (as NaF), 4.5 mg; iodine (as KIO_3), 5 mg; cobalt (as $CoCl_2.6H_2O$), 1 mg; selenium (as Na_2SeO_3), 0.10 mg (Hajen et al, 1993b).

Table 3.4.2.2 : Combination of ingredients for reference and test diets (on air-dry basis, expressed as percentage).

Ingredient	Refere -nce diet	Test diet (Fish meal)	Test diet (Sun -flower meal)
Basal diet	98.00	68.00	68.00
Fish meal (71.6% CP)	-	30.00	-
Sunflower meal (27.6% CP)	-	-	30.00
Chromic oxide	1.00	1.00	1.00
Micro tracer F-Ni	1.00	1.00	1.00
Total	100.00	100.00	100.00

Table 3.4.2.3 : Crude protein (N x 6.25), ash and marker contents in the experimental diets (expressed as % w/w as moisture free basis).

Component	Diets related with fish meal		Diets related with sunflower meal	
	Reference diet	Test diet	Reference diet	Test diet
Crude protein	60.00	63.94	55.31	50.63
Ash	9.03	11.68	9.36	9.76
Microtracer N-Fi	1.14	1.16	0.91	0.99
Chromic oxide	1.05	1.28	1.23	1.30
Acid insoluble ash	1.60	1.69	1.57	1.71

Specific growth rate (SGR) and food conversion ratio (FCR) were calculated by the following formula (Wee, 1983) :

$$SGR(\%) = \frac{\text{Ln of final body weight - Ln of initial body weight.}}{\text{Time (days)}}$$

$$FCR\ (\%) = \frac{\text{Weight of food presented (dry weight)}}{\text{Weight of fish produced (fresh weight)}}.$$

3.4.2.7 Statistical analyses

The apparent digestibility coefficient values were designed for statistical analysis by analysis of variance (ANOVA). Results were considered to be statistically significant if $P<0.05$. Prior to performing ANOVA, normality of the data were determined using Shapiro-Wilk W test; homogeneity of the variances was determined using Bartlett's test and Cochran's test. If the variances were

heterogeneous the data were transformed using 'arc sin square root'. When the obtained P value for the treatment or interaction in ANOVA were significant, determination of which pairs of groups were significant was made by multiple comparison of means using Fisher's LSD (Sokal and Rohlf, 1987). Student's t-test were used to compare two different means only. All the tests, except Cochran's, were made using JMP 2.0 computer package.

3.4.3 Results

3.4.3.1 Apparent digestibility coefficients of protein in Fish meal

The apparent digestibility coefficients (ADC) of protein in reference diet, test diet and fish meal ingredient estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers is shown in Figure 3.4.3.1. For all the markers the ADC of protein in test diet were always lower than that of reference diet, which decreased the ADC of protein values in fish meal ingredients proportionately. The ADC of protein in fish meal using the same three markers over four different days of collection is shown in Table 3.4.3.1. The analysis of variance for the ADC of protein in fish meal is shown in Table 3.4.3.2. The ADC values determined using microtracer F-Ni were relatively lower than using chromic oxide or acid insoluble ash as markers, although the differences were not significant ($P > 0.05$).

3.4.3.2 Apparent digestibility coefficients of dry matter in Fish meal

The apparent digestibility coefficients of dry matter in reference diet, test diet and fish meal ingredient estimated using three different markers is shown in Figure 3.4.3.2. The ADC of dry matter in test diets were higher than that of reference diets with chromic oxide and acid insoluble ash markers resulting in higher ADC values for the fish meal ingredients. The trends were opposite in case of microtracer F-Ni marker. The ADC of dry matter in fish meal using the same three markers over four different days of fecal collection is shown in Table 3.4.3.3. The analysis of variance for the ADC of dry

matter in fish meal is shown in Table 3.4.3.4. There were no significant difference (P>0.05) between the ADC values with different markers but the ADC of dry matter in fish meal using microtracer F-Ni as a marker were lower than both of the other markers.

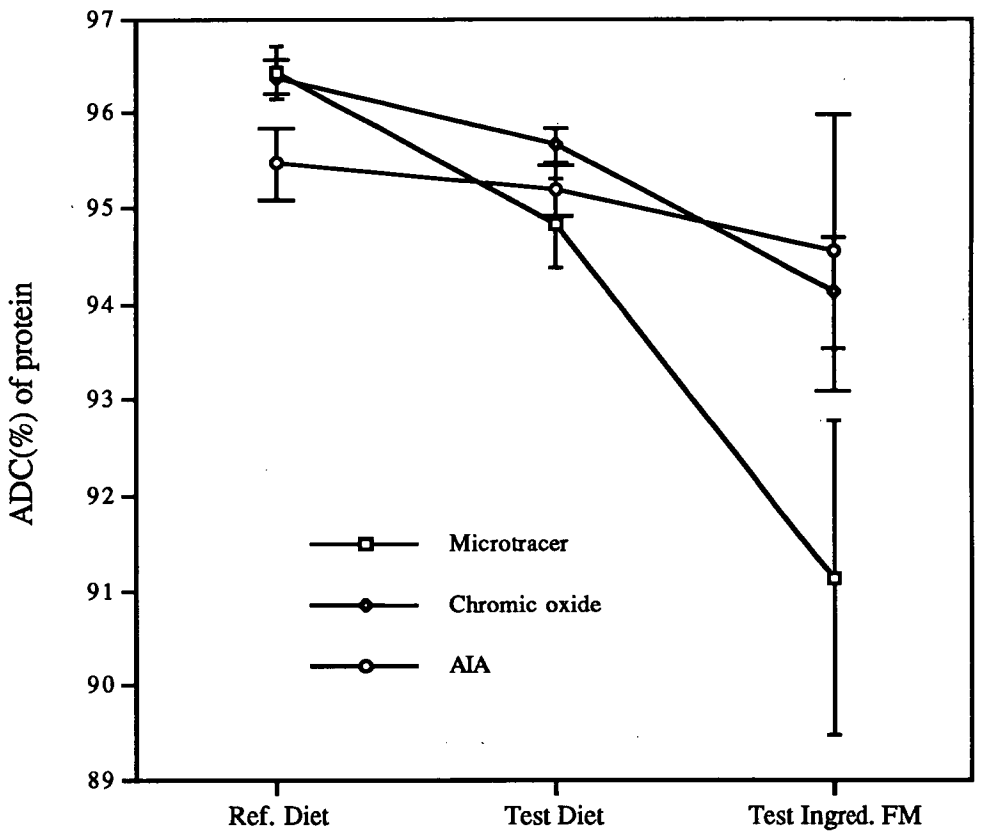


Figure 3.4.3.1 : Apparent digestibility coefficients (%) of protein in reference diet, test diet and test ingredient (Fish meal) estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers over 10 days of experimental period (mean ± SE, n = 8).

Table 3.4.3.1 : Apparent digestibility coefficients (%) of protein in Fish meal estimated using microtracer F-Ni, Chromic oxide, and acid insoluble ash as markers over four different days of fecal collection (mean ± SE, n = 2).

Days	ADC (%) of protein in Fish meal		
	Microtracer F-Ni marker	Chromic oxide marker	Acid insoluble ash marker
3	89.28 ± 3.03	94.09 ± 1.40	93.29 ± 4.85
5	90.84 ± 0.99	94.95 ± 0.18	94.34 ± 3.01
7	88.21 ± 4.41	95.07 ± 0.59	95.25 ± 1.28
10	96.18 ± 3.02	92.37 ± 1.59	95.27 ± 4.59
Total mean	91.13 ± 1.65	94.12 ± 0.58	94.54 ± 1.44

Table 3.4.3.2 : Analysis of variance for the apparent digestibility coefficients (%) of protein in fish meal under four different days of fecal collection and using three different markers.

Sources of variation	DF	SS	MS	F	P
Days	3	18.51	6.17	0.3739	0.7734
Markers	2	55.38	27.69	1.6777	0.2278
Days x markers	6	71.11	11.85	0.7181	0.6429
Error	12	198.05	16.50		
Total	23	343.05			

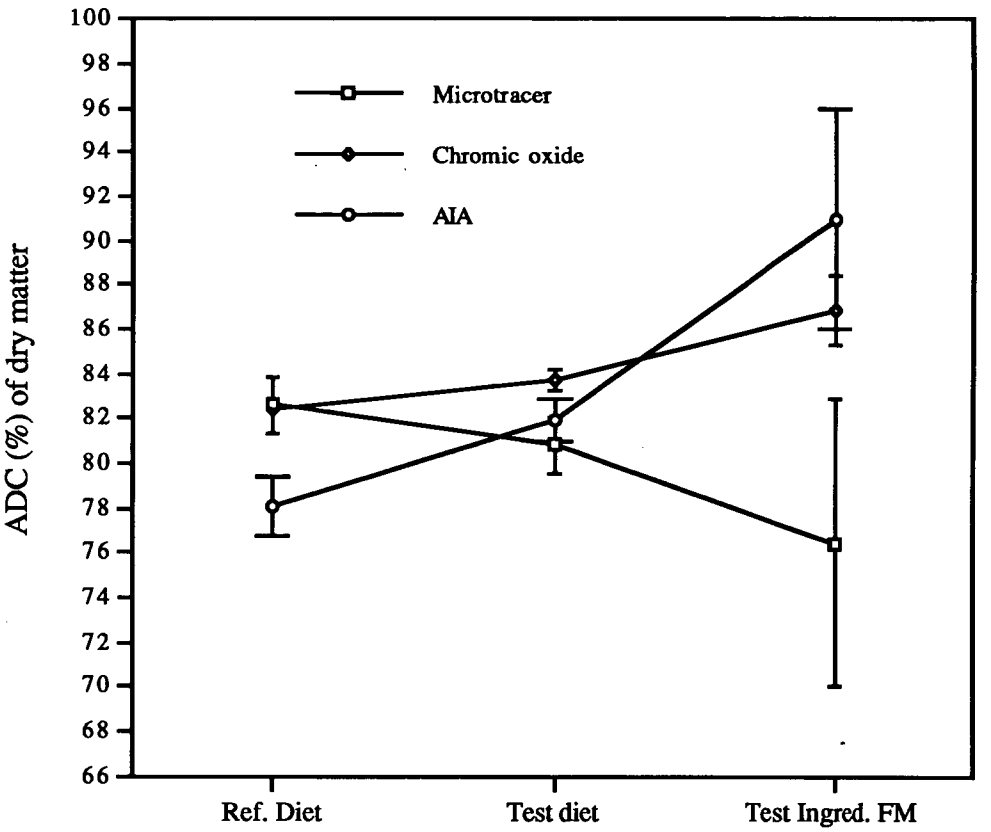


Figure 3.4.3.2 : Apparent digestibility coefficients of dry matter in reference diet, test diet and test ingredient (Fish meal) estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers over 10 days of experimental period (mean ± SE, n = 8).

Table 3.4.3.3 : Apparent digestibility coefficients (%) of dry matter in Fish meal estimated using microtracer F-Ni, chromic oxide, and acid insoluble ash as markers over four different days of fecal collection (mean ± SE, n = 2).

Days	ADC (%) of dry matter in Fish meal		
	Microtracer F-Ni marker	Chromic oxide marker	Acid insoluble ash marker
3	71.00 ± 12.97	89.46 ± 1.36	89.35 ± 17.23
5	73.07 ± 8.58	88.28 ± 2.30	88.69 ± 10.34
7	61.62 ± 4.47	86.12 ± 4.85	89.19 ± 1.98
10	100.04 ± 8.65	83.51 ± 3.69	96.68 ± 15.74
Total mean	76.43 ± 6.42	86.84 ± 1.52	90.97 ± 4.99

Table 3.4.3.4 : Analysis of variance for the apparent digestibility coefficients (%) of dry matter in Fish meal under four different days of fecal collection and using three different markers.

Sources of variation	DF	SS	MS	F	P
Days	3	674.78	224.93	1.3017	0.3190
Markers	2	898.65	449.33	2.6004	0.1153
Days x markers	6	1088.79	181.47	1.0502	0.4415
Error	12	2073.46	172.79		
Total	23	4735.68			

3.4.3.3 Apparent digestibility coefficients of protein in Sunflower meal

The apparent digestibility coefficients (%) of protein in reference diet, test diet, and sunflower meal ingredient estimated using chromic oxide, microtracer F-Ni, and acid insoluble ash (AIA) as markers is shown in Figure 3.4.3.3. The ADC values in test diet remained higher than that of reference diet using microtracer and chromic oxide marker resulting higher ADC values for test ingredient (Sunflower meal). But with the use of AIA marker, ADC values of protein for test diet as well as test ingredient decreased in comparison with reference diet. The ADC of protein in sunflower meal using the same three markers over four different days of fecal collection is shown in Table 3.4.3.5. Analysis of variance for the ADC of protein in sunflower meal is shown in Table 3.4.3.6. Although the ADC of protein with AIA marker appeared relatively lower than using other two markers, the differences were not significant ($P > 0.05$).

3.4.3.4 Apparent digestibility coefficients of dry matter in Sunflower meal

The apparent digestibility coefficients (%) of dry matter in reference diet, test diet, and sunflower meal ingredient estimated using chromic oxide, microtracer F-Ni, and acid insoluble ash (AIA) as markers is shown in Figure 3.4.3.4. The ADC values in test diet remained low than that of reference diet using all the markers resulting much lower ADC values for test ingredient (Sunflower meal). The ADC of dry matter in sunflower meal using the same

three markers over four different days of fecal collection is shown in Table 3.4.3.7. Analysis of variance for the ADC of protein in sunflower meal is shown in Table 3.4.3.8. Multiple comparison of the means of ADC values using three different markers is shown in Figure 3.4.3.5. The ADC values using microtracer as a marker were significantly lower ($P < 0.05$) than that using acid insoluble ash a marker.

3.4.3.5 Growth performance of the fish with Fish meal and Sunflower meal ingredients

The effect of reference diet and test diet with sunflower meal and fish meal are shown in Table 3.4.3.9 and Table 3.4.3.10. In case of sunflower meal, weight gain, % weight gain, SGR, FCR were not significantly different ($P>0.05$) with the corresponding reference diet, although the fish fed reference diet had higher SGR values and lower FCR values than the corresponding sunflower meal test diet. Weight gain and FCR values were significantly improved ($P<0.05$) in fish meal diet in relation to the corresponding reference diet.

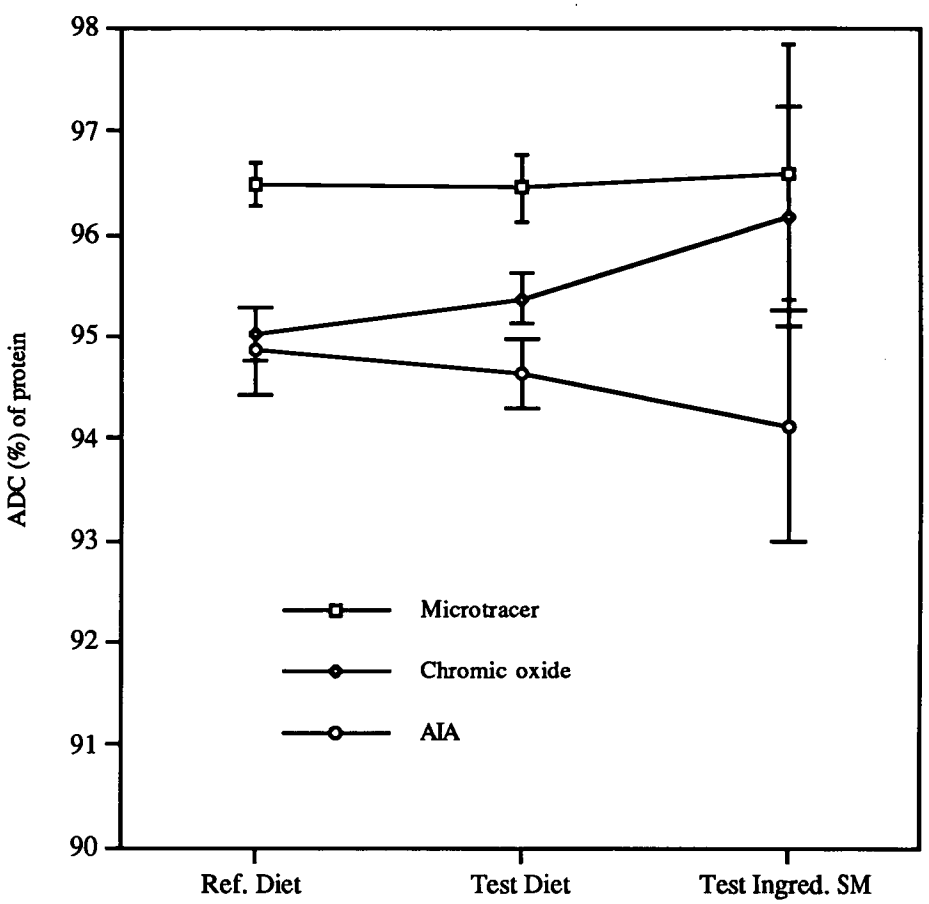


Figure 3.4.3.3 : Apparent digestibility coefficients (%) of protein in reference diet, test diet, Sunflower meal ingredient estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers over 10 days of experimental period (mean ± SE, n = 8).

Table 3.4.3.5 : Apparent digestibility coefficients (%) of protein in Sunflower meal estimated using microtracer F-Ni, Chromic oxide, and acid insoluble ash as markers over four different days of fecal collection (mean ± SE, n = 2).

Days	ADC (%) of protein in Sunflower meal		
	Microtracer F-Ni marker	Chromic oxide marker	Acid insoluble ash marker
3	96.95 ± 2.38	95.17 ± 1.58	96.66 ± 3.77
5	92.13 ± 2.72	96.72 ± 1.25	92.92 ± 3.38
7	98.42 ± 0.08	96.93 ± 0.26	93.79 ± 1.08
10	98.81 ± 1.11	95.85 ± 5.02	93.11 ± 0.41
Total mean	96.60 ± 1.24	96.17 ± 1.06	94.12 ± 1.13

Table 3.4.3.6 : Analysis of variance for the apparent digestibility coefficients (%) of protein in Sunflower meal under four different days of fecal collection and using three different markers.

Sources of variation	DF	SS	MS	F	P
Days	3	23.77	7.92	0.6738	0.5845
Markers	2	27.73	13.87	1.1793	0.3407
Days x markers	6	54.94	9.16	0.7788	0.6021
Error	12	141.09	11.76		
Total	23	247.53			

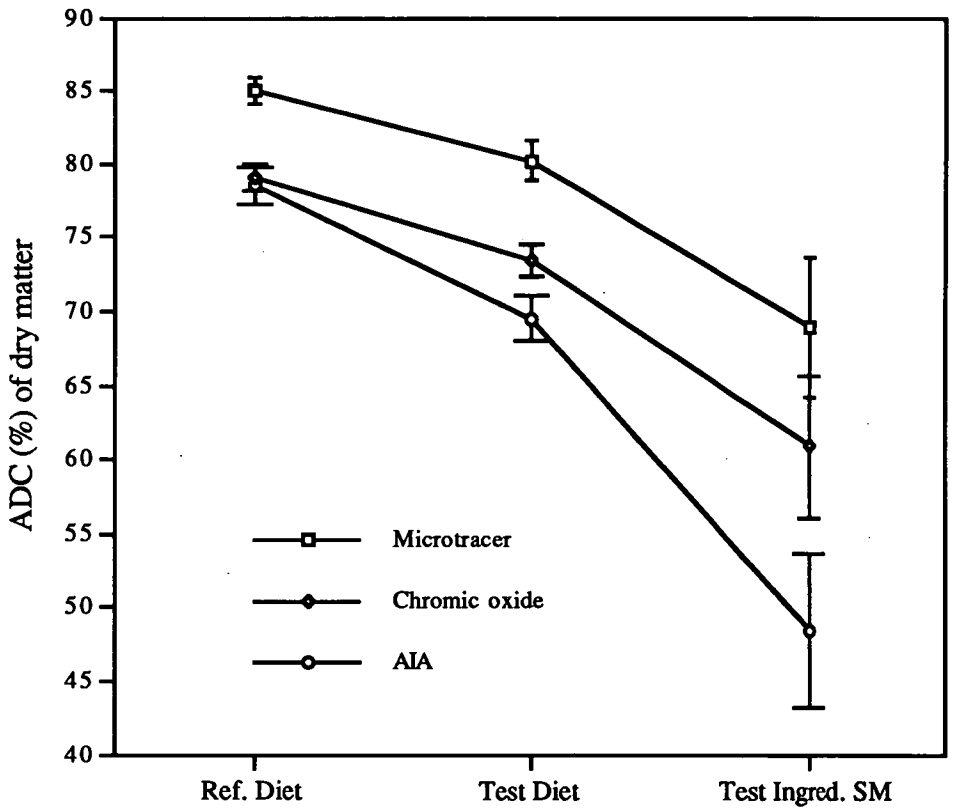


Figure 3.4.3.4 : Apparent digestibility coefficients (%) of dry matter in reference diet, test diet and test ingredient (Sunflower meal) estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers over 10 days of experimental period (mean ± SE, n = 8).

Table 3.4.3.7 : Apparent digestibility coefficients (%) of dry matter in Sunflower meal estimated using microtracer F-Ni, chromic oxide, and acid insoluble ash as markers over four different days of fecal collection (mean ± SE, n = 2).

Days	ADC (%) of dry matter in Sunflower meal		
	Microtracer F-Ni marker	Chromic oxide marker	Acid insoluble ash marker
3	67.83 ± 11.98	55.35 ± 6.47	62.07 ± 15.40
5	52.54 ± 4.14	69.46 ± 5.72	53.22 ± 5.20
7	75.21 ± 1.77	59.52 ± 0.61	36.16 ± 4.79
10	80.13 ± 4.26	59.09 ± 21.6	42.31 ± 8.31
Total mean	68.93 ± 4.69	60.85 ± 4.82	48.44 ± 5.19

Table 3.4.3.8 : Analysis of variance for the apparent digestibility coefficients (%) of dry matter in Sunflower meal under four different days of fecal collection and using three different markers.

Sources of variation	DF	SS	MS	F	P
Days	3	82.42	27.47	0.1527	0.9260
Markers	2	1704.12	852.06	4.735	0.0305
Days x markers	6	1798.93	299.82	1.666	0.2126
Error	12	2159.38	179.95		
Total	23	5744.85			

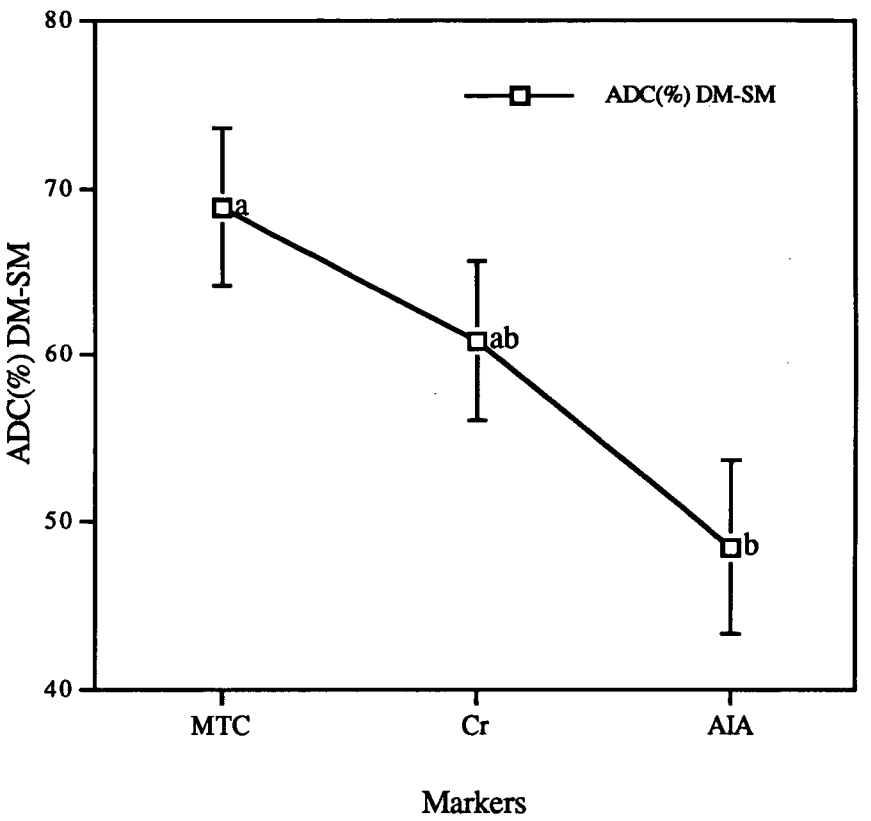


Figure 3.4.3.5 : Apparent digestibility coefficients (%) of dry matter in Sunflower meal estimated using microtracer F-Ni, chromic oxide and acid insoluble ash as markers (mean ± SE, n = 8). Mean values sharing same superscripts are not significantly different (P > 0.05).

Table 3.4.3.9 : Effect of reference diet and test diet (with Sunflower meal) (± SE.) on feed intake, specific growth rate and food conversion ratio of rainbow trout, *O. mykiss* over a period of 10 days.

Components	Reference diet	Test diet (Sunflower meal)
Initial weight (g).	235.51 ± 9.16	221.15 ± 8.66
Final weight (g).	275.30 ± 11.57	248.46 ± 10.44
Weight gain (g).	39.79 [a1] ± 3.11	27.31 [a] ± 6.09
% weight gain .	16.93 [a] ± 1.61	12.22 [a] ± 1.92
Feed intake (% b.w./ day)	0.202 [a] ± 0.004	0.191 [a] ± 0.013
SGR (% / day)	1.56 [a] ± 0.14	1.15 [a] ± 0.17
FCR	0.92 [a] ± 0.07	1.25 [a] ± 0.31

[1] Within each row, values with a common superscript are not significantly different (P > 0.05).

Table 3.4.3.10 : Effect of reference diet and test diet (with Fish meal ± SE.) on feed intake, specific growth rate and food conversion ratio of rainbow trout, *O. mykiss* over a period of 10 days.

Components	Reference diet	Test diet (Fish meal)
Initial weight (g).	275.30 ± 11.57	248.46 ± 10.44
Final weight (g).	307.73 ± 12.64	290.15 ± 10.82
Weight gain (g).	32.44 [a1] ± 0.49	41.70 [b] ± 1.05
% weight gain .	11.78 [a] ± 0.14	16.94 [a] ± 1.85
Feed intake (% b.w./ day)	0.197 [a] ± 0.001	0.199 [a] ± 0.017
SGR (% / day)	1.12 [a] ± 0.02	1.57 [a] ± 0.16
FCR	1.29 [a] ± 0.02	0.91 [b] ± 0.03

[1] Within each row, values with a common superscript are not significantly different (P > 0.05).

3.4.4 Discussion

The apparent protein and dry matter digestibility coefficients of fish meal ingredient using microtracer F-Ni, chromic oxide and acid insoluble ash as markers were not significantly ($P > 0.05$) different (Tables 3.4.3.1 to 3.4.3.4). But the digestibility coefficients of protein in fish meal estimated using microtracer F-Ni marker were 3.28 and 3.74 percentage units lower than using chromic oxide and acid insoluble ash markers respectively. Similar trends for dry matter digestibility coefficients in fish meal were also observed with microtracer F-Ni, which resulted in the ADC values 13.62 and 19.02 percentage units lower than chromic oxide and acid insoluble ash markers respectively.

Although the acid insoluble ash resulted higher digestibility coefficients but from statistical point of view this difference might be accepted as similar ($P>0.05$). This findings were different from the findings of previous Experiment 3. The possible explanations for this were three : 1) the fish were fed twice a day in his experiment while in the previous experiment it was once a day. There might be some relation between the feeding level and digestibility of nutrients. Henken et al. (1985) found that apparent digestibility of protein and dry matter by African catfish (*Clarias gariepinus*) was negatively correlated with feeding level. Windell et al. (1978) found the similar results for dry matter digestibilities but they could not find variations in the digestibility of protein and lipid by rainbow trout with increasing feeding levels. At higher feeding levels the passage rate of the dietary material through the digestive tract is thought to be higher, causing less material to be digested or absorbed. 2) The another possible cause might be the larger size of the fish in this

experiment than the previous experiments. Hepher (1988) mentioned that the enzymatic activity may vary with fish age which may affect digestibility coefficients. Windell et al. (1978) found that the nutrient digestibilities in smaller sized fish (18.6 g) were significantly lower when compared with medium (207.1 g) or large (585.7 g) sized fish but only at low temperatures (7°C). 3) Last but most important possibilities are the use of different feed. In Experiment 4 commercial trout pellet were used but in this experiment diets were prepared in the laboratory. There might be a difference between source and composition of the feedstuff and the processing technique in treating the ingredients. Differences in the manufacturing process employed in the preparation of diet can cause variation in digestibilities (Tacon, 1990).

The ADC of protein in fish meal ranged 91.13 to 94.54 percentage units by using three different markers. This results were in the range of 83.1 to 93.6 percentage units which was described by Hajen et al. (1993) for the ADC of different kinds of fish meal. Cho et al. (1985) indicated that the nutrient digestibility coefficients of fish meal would vary considerably according to source, composition and processing methods. The ADC of dry matter in fish meal ingredient were lowest (76.43%) for microtracer marker and highest for acid insoluble ash marker (90.97%) . In between these two levels, was the ADC of dry matter by chromic oxide marker (86.84%). Cho et al. (1985) reported the dry matter digestibility coefficient of Herring fish meal as 85% measured with chromic oxide marker. The ADC of dry matter as measured with chromic oxide marker in this experiment were in close agreement with the results of Cho et al. (1985). However as the lowest and highest ADC values for dry

matter with microtracer F-Ni and acid insoluble ash markers in this experiment were not significantly different ($P > 0.05$), it might be considered as similar with the published information.

The ADC of protein in Sunflower meal ingredient using microtracer F-Ni, chromic oxide and acid insoluble ash, ranged from 94.12 to 96.60%, were not significantly ($P > 0.05$) different (Tables 3.4.3.5 and 3.4.3.6). This ADC values obtained for Sunflower meal in this experiment were in contrary with the findings of Tacon et al. (1984) who found the ADC of sunflower meal was 79.2% (incorporation of sunflower meal was 36.5% of the diet) with rainbow trout. In their experiment, they used very smaller sized fish (5.8 g). Kitamikada and Tachino (1960, as cited by Wee, 1992) reported that digestive capabilities increased with the age of the fish. Possibly the high digestibility coefficient of protein in sunflower meal in this experiment was due to the age and size of the fish. However, most importantly, the discrepancy is most likely due to different diet composition.

The dry matter digestibility of Sunflower meal ingredient using the three markers (68.93, 60.85 and 48.44 percents with microtracer F-Ni, chromic oxide and AIA respectively) were significantly different ($P<0.05$). However, digestibility coefficients using microtracer F-Ni marker were not significantly different with using chromic oxide marker (Figure 3.4.3.5). Tacon et al. (1984) reported ADC of dry matter of Sunflower meal as 42.9%. The higher ADC values in this experiment might be due to the difference of diet composition, difference of fish size and possibly with the difference of environmental temperature. During this study the water was 9°C-

13.9°C while in Tacon et al's (1984) study average temperature was 8.8°C.

The growth performances for Fish meal and Sunflower meal in relation to SGR, FCR indicated that the experimental diets supported good growth performances in comparison to the reference diet. As the length of the experiments were too short to study the growth parameters in details, no comparisons were attempted with published information on growth parameters using same ingredients.

The ADC values generated by using microtracer F-Ni and chromic oxide markers in this experiment were quite consistent and comparable. Acid insoluble ash also produce comparable results with either microtracer F-Ni or chromic oxide except in dry matter estimation for sunflower meal. The performance of microtracer F-Ni as an external marker in digestibility studies followed the results obtained in Experiment 4. Therefore, it might be concluded that microtracer F-Ni is suitable as an external marker for digestibility studies with either ingredients or diets in rainbow trout.

4.1 Final summary and conclusion

It is clear from the results of this study is that the use of microtracer F-Ni as an external marker is quite acceptable in mixing efficiency testing and nutrient digestibility coefficients estimation. The methods used in measuring microtracer F-Ni in digestibility studies is easy in comparison with the use of chromic oxide. For chromic oxide analysis, analytical machinery and laboratory facilities are required for chromium determination. In addition, the reagents used are considered as dangerous as it has explosive properties (Bolin et al., 1952) and therefore requiring technicians to be highly skilled and trained. The number of laboratories conducting chromium determination are very few, as result, it may take a long time to get the results of analysis, which prevents quick in action for food ingredient selection at feed mill site. On the other hand determination of microtracer F-Ni is simple, easy and could be performed in the field site with very few special needs. The technician doesn't need any special training to conduct this analysis.

At present, chromic oxide is the most commonly used external marker for digestibility studies in fish or in other animals. The different digestibility values generated in this study using microtracer F-Ni as external marker are quite consistent and comparable with those produced by using chromic oxide as an external marker in all the specific situations. The use of acid insoluble ash as a marker resulted in inconsistent ADC values when compared with either chromic oxide or microtracer F-Ni as a marker. The digestibility values calculated using AIA were found to

be different under different culture situations and with different diets. Several authors (Tacon and Rodrigues, 1984; De Silva and Perera, 1983) encountered this sort of problem when using acid insoluble ash as a marker in digestibility studies. Therefore, it is concluded that in this study, the performance of acid insoluble ash as a marker in digestibility coefficients determination were very poor.

On the other hand ADC values determined using microtracer F-Ni as a marker at 1% inclusion level are consistent and comparable with the results calculated by using widely used and accepted chromic oxide marker at 1% level. Considering this consistency of digestibility results and easy methods of determination it is concluded that microtracer F-Ni at 1% inclusion level could be useful as external marker in digestibility studies in rainbow trout.

4.2 Problems encountered in this study

The microtracer F-Ni used in this study were supplied by Micro Tracers, Inc., USA and the particles were not of a uniform size (Experiment 1). Although efforts were made to establish an average uniform particle counts per unit weight in this study, it is highly desirable that the particle size should be uniform to reduce variation in the concentrations of microtracer measured and improve consistency of results.

Acid insoluble ash contents from diets incorporated with microtracer F-Ni were higher than values obtained with chromic oxide containing diets. This two markers were incorporated into the same basic diet at same concentration. Theoretically, the acid insoluble ash values should be same for both the experimental diets. The possible explanation were loss of chromium in high temperature (Experiment 3). However, it is not known whether iron-nickel particles are oxidised in high temperature resulting in higher AIA values. Therefore, if AIA is to be used as a marker, it is important to know the effects of the ashing and acid hydrolysis procedure used in the analysis, on the ingredients.

During the retrieval procedure of the microtracer particles from the feed and fecal samples, there might be some loss of particles at this point. In this study no effort were made to quantify such losses of particles or to determine whether there were any losses at all or not.

The different ADC values calculated using microtracer F-Ni showed a wider variation of standard errors in comparison with ADC values

using chromic oxide as a marker. This variation may possibly be due to the non uniformity of particle size or possible loss of particles during retrieval procedure as described before.

4.3 Suggestions for future work

It has been established in this study that microtracer F-Ni at 1% inclusion level could be used as an external marker for digestibility studies in rainbow trout. It would be highly desirable to check the suitability of this marker in digestibility estimations with other fish species.

It has been shown that the use of microtracer F-Ni resulted in no short term adverse effect on the physiology of fish (as the fish were healthy and grew well). To be an ideal marker, it should not have any adverse effect on the life of the animal. Therefore a long term study would be beneficial to test any possible physiological effect (growth, reproduction, deformities of internal organ and tissues etc) of this tracer on the fish.

In the present study no efforts were made to test whether there are any loss or absorption of microtracer F-Ni particles within the fish body. It would be beneficial to test this by using radioactive tracer particles in the diet and subsequently analysing the different organs or tissues of the fish body for the determination of radio-active materials if presents or not. This study will test the basic stipulation of using marker in digestibility studies, ie., the marker should not be digested or absorbed.

Recovery of particles from different parts of the digestive tract at 1% inclusion showed a progressive, sequential recovery pattern. This kind of results were expected, at least theoretically. But it is essential to measure the exact flow rate and kinetics of food particles and microtracer particles along the digestive tract because a identical velocity of these two are necessary to become a good marker.

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Appendices

Appendix One

(Experiment Two)

Appendix 1.2.1 : Microtracer F-Ni recovery (% w/w) from different parts of the digestive tract (Day 1)				
Treat. Level	Stomach	Intestine	Rectum	Feces
0.25	0.1738	0.2617	0.3540	0.3600
0.25	0.0996	0.1230	0.4641	
0.25	0.4076	0.3483	0.8566	0.2271
0.25	0.4410	0.4261	0.9952	
0.25	0.2076	0.2165	0.3011	0.5929
0.25	0.0596	0.4849	0.9002	
Mean	0.2315	0.3101	0.6452	0.3933
SD	0.1586	0.1353	0.3060	0.1852
SE	0.0647	0.0553	0.1249	0.1069
0.5	0.3139	1.1104	1.1405	2.1476
0.5	0.4704	0.3987	1.4358	
0.5	0.5086	1.1161	2.0180	1.2397
0.5	0.6258	1.3653	2.8533	
0.5	0.7788	0.3204	1.1527	1.6658
0.5	0.5154	0.5818	0.3634	
Mean	0.5355	0.8154	1.4940	1.6844
SD	0.1561	0.4366	0.8539	0.4542
SE	0.0637	0.1782	0.3486	0.2623
1	0.6276	1.1914	1.6485	3.2469
1	1.1790	1.3318	2.7031	
1	1.2868	0.8285	1.4205	2.0546
1	1.0367	1.1650	0.9118	
1	1.4098	3.2037	4.4545	2.6264
1	1.2597	2.6408	3.1250	
Mean	1.1333	1.7269	2.3772	2.6426
SD	0.2768	0.9573	1.3095	0.5963
SE	0.1130	0.3908	0.5346	0.3443
2	0.1319	1.5032	2.7282	-
2	1.1359	0.3685	1.3718	
2	2.3371	3.9553	1.9003	4.2681
2	0.5021	3.0025	2.4124	
2	2.6500	2.0702	1.9393	4.6561
2	1.1646	0.9926	2.1711	
Mean	1.3203	1.9820	2.0872	4.4621
SD	0.9942	1.3232	0.4674	0.2744
SE	0.4059	0.5402	0.1908	0.1940

Appendix 1.2.2 : Microtracer F-Ni recovery (% w/w) from different parts of the digestive tract (Day 2)				
Treat. Level	Stomach	Intestine	Rectum	Feces
0.25	0.2078	-	0.4908	0.4955
0.25	0.1347	0.5284	0.1708	
0.25	0.3582	0.4784	0.2873	0.3622
0.25	0.2387	0.1695	0.7772	
0.25	0.4473	0.5805	0.3074	0.4370
0.25	0.2131	0.4121	0.1720	
Mean	0.2666	0.4338	0.3676	0.4316
SD	0.1145	0.1603	0.2324	0.0668
SE	0.0467	0.0717	0.0949	0.0386
0.5	0.4878	1.6667	2.9920	0.7905
0.5	0.3809	0.4631	0.8146	
0.5	0.4629	0.2399	0.6378	0.6944
0.5	0.4419	0.5590	1.0371	
0.5	0.5520	0.1344	4.0643	0.6132
0.5	0.1899	0.2633	0.2663	
Mean	0.4191	0.5544	1.6354	0.6994
SD	0.1255	0.5667	1.5259	0.0888
SE	0.0512	0.2313	0.6229	0.0512
1	0.2708	1.0832	1.1461	2.0944
1	0.9786	0.9889	2.0833	
1	1.0673	1.7756	2.8362	1.3979
1	1.2970	0.5672	1.7973	
1	0.7440	1.5210	0.8778	1.9804
1	0.5798	1.5354	0.8121	
Mean	0.8229	1.2452	1.5921	1.8242
SD	0.3685	0.4457	0.7936	0.3736
SE	0.1504	0.1820	0.3240	0.2157
2	0.6745	0.2367	1.3089	2.5681
2	0.4248	1.0632	0.9802	
2	1.2193	0.9365	1.5067	6.3598
2	0.3334	1.4341	1.5625	
2	2.1802	0.3950	2.9030	2.2582
2	2.0115	3.6946	1.4685	
Mean	1.1406	1.2934	1.6216	3.7287
SD	0.8033	1.2562	0.6620	2.2839
SE	0.3280	0.5128	0.2703	1.3186

Appendix 1.2.3 : Microtracer F-Ni recovery (% w/w) from different parts of the digestive tract (Day 3)				
Treat. Level	Stomach	Intestine	Rectum	Feces
0.25	0.6696	1.8141	0.7517	0.4053
0.25	0.6505	1.4205	3.2106	
0.25	0.1479	0.8013	1.2738	0.3219
0.25	0.9546	0.5849	0.3389	
0.25	0.2541	2.5123	1.0098	0.4271
0.25	0.2699	0.5040	0.8754	
Mean	0.4911	1.2729	1.2434	0.3848
SD	0.3146	0.7922	1.0120	0.0555
SE	0.1285	0.3234	0.4131	0.0321
0.5	0.6192	0.9348	2.4740	1.5503
0.5	0.6932	1.5052	5.6818	
0.5	0.7238	0.8030	2.7528	1.7368
0.5	0.6811	0.8755	1.9634	
0.5	0.7140	0.3524	6.3291	1.2563
0.5	1.0730	0.8446	1.7361	
Mean	0.7507	0.8859	3.4895	1.5145
SD	0.1621	0.3686	1.9923	0.2422
SE	0.0662	0.1505	0.8134	0.1399
1	1.0911	1.6650	2.5436	4.3308
1	0.7345	2.2197	5.4745	
1	4.2279	3.7276	6.2866	3.9149
1	1.6371	3.8265	2.5568	
1	0.8956	1.5879	2.5240	3.8746
1	2.8324	2.6042	2.5568	
Mean	1.9031	2.6052	3.6571	4.0401
SD	1.3696	0.9817	1.7414	0.2526
SE	0.5591	0.4008	0.7109	0.1458
2	2.9186	4.0732	5.3439	7.3357
2	1.9336	4.0594	2.9122	
2	-	2.1143	3.2475	4.3408
2	1.1427	2.3304	3.9336	
2	2.2321	1.4628	6.2500	7.5622
2	1.6374	5.8798	11.9792	
Mean	1.9729	3.3200	5.6111	6.4129
SD	0.6642	1.6461	3.3680	1.7981
SE	0.2971	0.6720	1.3750	1.0381

Appendix Two

(Experiment Three)

Appendix 2. 3/1. 1 : Recovery of microtracer (% w/w) from diet, rectum (stripping) and feces (expt. 3/1)					
		6 hours	interval	24 hours	interval
Day	Replication	Stripping	Faecal collection	Stripping	Faecal collection
3	1	2.8193	1.7974	1.7383	3.7023
	2	2.1742	3.1888	2.7872	3.5457
	Mean	2.4968	2.4931	2.2628	3.6240
	Std	0.46	0.98	0.74	0.11
	S.E.	0.32	0.70	0.52	0.08
5	1	2.9751	2.9436	1.6919	3.7333
	2	1.5160	2.3256	2.1551	3.6085
	Mean	2.2456	2.6346	1.9235	3.6709
	Std	1.03	0.44	0.33	0.09
	S.E.	0.73	0.31	0.23	0.06
7	1	2.3209	3.6706	2.5868	3.0585
	2	2.0715	2.2660	2.4788	4.1170
	Mean	2.1962	2.9683	2.5328	3.5878
	Std	0.18	0.99	0.08	0.75
	S.E.	0.12	0.70	0.05	0.53
14	1	2.3602	2.3736	2.3643	2.5989
	2	3.3283	2.6027	1.6991	2.7029
	Mean	2.8443	2.4882	2.0317	2.6509
	Std	0.68	0.16	0.47	0.07
	S.E.	0.48	0.11	0.33	0.05
Overall Mean		2.4457	2.6460	2.1877	3.3834
Overall S.E.		0.20	0.21	0.15	0.19
Microtracer recovery from diet.					
	Replication	% w/w basis			
	1	0.7745			
	2	0.9521			
	3	1.0262			
	Mean	0.9176			
	S.E.	0.07			

Appendix 2. 3/1. 2 : Recovery of chromic oxide (% w/w) from the contents of rectum (stripping), feces and diet in experiment 3/1.					
		6 hours	interval	24 hours	interval
Day	Replication	Stripping	Fecal collection	Stripping	Fecal collection
3	1	2.5404	3.5770	3.3580	4.8472
	2	2.8908	3.7084	2.6718	4.3654
	Mean	2.7156	3.6427	3.0149	4.6063
	Std	0.25	0.09	0.49	0.34
	S.E.	0.18	0.07	0.34	0.24
5	1	3.1536	2.8908	3.2412	4.1026
	2	4.1902	4.2632	3.0076	4.5260
	Mean	3.6719	3.5770	3.1244	4.3143
	Std	0.73	0.97	0.17	0.30
	S.E.	0.52	0.69	0.12	0.21
7	1	2.5112	3.5478	2.9784	4.4092
	2	2.5696	3.6062	3.2120	3.8982
	Mean	2.5404	3.5770	3.0952	4.1537
	Std	0.04	0.04	0.17	0.36
	S.E.	0.03	0.03	0.12	0.26
14	1	4.0150	3.9858	3.0806	4.6282
	2	3.7230	3.8836	3.3142	4.7742
	Mean	3.8690	3.9347	3.1974	4.7012
	Std	0.21	0.07	0.17	0.10
	S.E.	0.15	0.05	0.12	0.07
Overall Mean		3.1992	3.6829	3.1080	4.4439
Overall S.E.		0.24	0.14	0.08	0.11
Chromic oxide recovery from diet.					
	Replication	% (w/w basis)			
	1	1.4746			
	2	1.2994			
	3	1.0220			
	Mean	1.2653			
	S.E.	0.13			

Appendix 2. 3/1. 3 : Protein content (%) of diet and feces at different time intervals and days (experiment 3/					
		6 hours	interval	24 hours	interval
Day	Replication	With Cr oxide	With F-Ni	With Cr oxide	With F-Ni
3	1	15.06	16.06	15.38	13.94
	2	15.69	14.19	15.31	14.38
	Mean	15.38	15.13	15.35	14.16
	S.E.	0.32	0.94	0.03	0.22
5	1	12.88	15.38	14.19	16.81
	2	14.94	16.94	16.63	14.06
	Mean	13.91	16.16	15.41	15.44
	S.E.	1.03	0.78	1.22	1.37
7	1	14.88	15.13	16.25	14.81
	2	15.75	16.06	15.25	15.31
	Mean	15.32	15.60	15.75	15.06
	S.E.	0.43	0.46	0.50	0.25
14	1	16.88	15.50	13.44	15.44
	2	16.81	16.69	14.00	13.19
	Mean	16.85	16.10	13.72	14.32
	S.E.	0.04	0.59	0.28	1.13
Overall mean		15.36	15.74	15.06	14.74
Overall S.E.		0.45	0.31	0.39	0.40
	Protein content (%) in diets.				
	Replication	D-1 (Cr-oxide)	D-2(F-Ni)		
	1	50.19	53.88		
	2	49.38	52.50		
	3	50.44	50.13		
	Mean	50.00	52.17		
	S.E.	0.32	1.09		

Appendix 2. 3/1. 4 : Protein content (%) of rectum content at different time intervals and days (experiment 3/1)					
		6 hours	interval	24 hours	interval
Day	Replication	With Cr oxide	With F-Ni	With Cr oxide	With F-Ni
3	1	14.44	16.69	16.50	14.75
	2	15.94	18.00	16.69	15.50
	Mean	15.19	17.35	16.60	15.13
	Std	1.06	0.93	0.13	0.53
	S.E.	0.75	0.65	0.09	0.38
5	1	14.63	17.75	15.00	13.50
	2	17.19	17.56	15.56	14.56
	Mean	15.91	17.66	15.28	14.03
	Std	1.81	0.13	0.40	0.75
	S.E.	1.28	0.09	0.28	0.53
7	1	16.94	17.88	13.69	14.75
	2	17.19	16.88	14.44	13.44
	Mean	17.07	17.38	14.07	14.10
	Std	0.18	0.71	0.53	0.93
	S.E.	0.12	0.50	0.38	0.65
14	1	15.19	17.38	14.75	15.13
	2	15.00	17.44	17.44	15.81
	Mean	15.10	17.41	16.10	15.47
	Std	0.13	0.04	1.90	0.48
	S.E.	0.10	0.03	1.34	0.34
Overall mean		15.82	17.45	15.51	14.68
Overall S.E.		0.41	0.16	0.45	0.30

Appendix 2. 3/1. 5 : Ash content (%) of diet and feces at different time intervals and days (experiment 3/1).					
		6 hours	interval	24 hours	interval
Day	Replication	With Cr oxide	With F-Ni	With Cr oxide	With F-Ni
3	1	21.9126	25.9618	21.1662	22.9879
	2	22.5575	28.1437	21.1311	24.4651
	Mean	22.2351	27.0528	21.1487	23.7265
	S.E.	0.32	1.09	0.02	0.74
5	1	22.1275	26.3294	21.0256	27.7223
	2	20.6979	27.3670	23.8629	26.2388
	Mean	21.4127	26.8482	22.4443	26.9806
	S.E.	0.71	0.52	1.42	0.74
7	1	21.6207	29.3881	25.5344	27.9878
	2	21.1399	-	22.2851	27.0756
	Mean	21.3803	29.3881	23.9098	27.5317
	S.E.	0.24	0.00	1.62	0.46
14	1	22.6893	29.9151	23.9236	28.8179
	2	24.2619	30.8826	20.8692	28.7919
	Mean	23.4756	30.3989	22.3964	28.8000
	S.E.	0.79	0.48	1.53	0.01
Overall Mean		22.1259	28.2840	22.4748	26.7609
Overall S.E.		0.39	0.70	0.62	0.74
	Ash content (%) in diets.				
	Replication	D-1 (Cr-oxide)	D-2(F-Ni)		
	1	9.1958	10.4076		
	2	9.4272	10.3979		
	3	9.4418	9.9338		
	4	-	10.1899		
	Mean	9.3549	10.2323		
	Std	0.14	0.22		
	S.E.	0.08	0.11		

Appendix 2. 3/1. 6 : AIA content (%) of diet and feces at different time intervals and days (experiment 3/1).					
		6 hours	interval	24 hours	interval
Day	Replication	With Cr oxide	With F-Ni	With Cr oxide	With F-Ni
3	1	3.5372	3.9132	4.3405	4.2077
	2	3.1511	9.2487	4.9379	5.1573
	Mean	3.3442	6.5810	4.6392	4.6825
	S.E.	0.19	2.67	0.30	0.47
5	1	2.5694	5.0647	3.7246	7.2338
	2	4.1541	8.7158	3.1771	6.1880
	Mean	3.3618	6.8903	3.4509	6.7109
	S.E.	0.79	1.83	0.27	0.52
7	1	4.5260	8.7473	2.8625	6.6258
	2	3.2755	4.6119	4.3892	-
	Mean	3.9008	6.6796	3.6259	6.6258
	S.E.	0.63	2.07	0.76	-
14	1	4.6014	6.2992	4.2833	4.6966
	2	3.2208	7.9886	2.4063	7.2133
	Mean	3.9111	7.1439	3.3448	5.9550
	S.E.	0.69	0.84	0.94	1.26
Over all Mean		3.6294	6.7899	3.7652	5.9032
Over all S.E.		0.26	0.74	0.31	0.46
AIA content (%) in diets.					
	Replication	D-1 (Cr-oxide)	D-2(F-Ni)		
	1	0.3927	1.4444		
	2	0.5482	1.5389		
	3	0.4695	1.4457		
	4	0.4919	1.4889		
	5	0.5343	-		
	Mean	0.4873	1.4795		
	S.E.	0.03	0.02		

Appendix 2.3/1.7 : ADC (%) of protein using chromic oxide as marker (expt. 3/1).					
		6 hours interval		24 hours interval	
Days	Replication	Stripping	Fecal collection	Stripping	Fecal collection
3	1	85.62	89.35	87.57	91.97
	2	86.05	89.29	84.19	91.12
	Mean	85.84	89.32	85.88	91.55
	Std	0.30	0.04	2.39	0.60
	S.E.	0.21	0.03	1.69	0.43
5	1	88.26	88.72	88.29	91.25
	2	89.62	91.13	86.91	90.70
	Mean	88.94	89.93	87.60	90.98
	Std	0.96	1.70	0.98	0.39
	S.E.	0.68	1.20	0.69	0.27
7	1	82.93	89.39	88.37	90.67
	2	83.07	88.95	88.62	90.10
	Mean	83.00	89.17	88.50	90.39
	Std	0.10	0.31	0.18	0.40
	S.E.	0.07	0.22	0.12	0.28
14	1	90.43	89.28	87.88	92.65
	2	89.80	89.05	86.68	92.58
	Mean	90.12	89.17	87.28	92.62
	Std	0.45	0.16	0.85	0.05
	S.E.	0.31	0.11	0.60	0.03
Overall Mean		86.97	89.40	87.31	91.38
Over all standard dev		3.00	0.74	1.44	0.93
Over all standard error		1.06	0.26	0.51	0.33

Appendix 2.3/1.8 : ADC (%) of protein using microtracer F-Ni as a marker (expt. 3/1).					
		6 hours interval		24 hours interval	
Days	Replication	Stripping	Faecal collection	Stripping	Faecal collection
3	1	89.59	84.28	85.08	93.38
	2	85.44	92.17	90.22	92.87
	Mean	87.52	88.23	87.65	93.13
	Std	2.93	5.58	3.63	0.36
	S.E.	2.08	3.95	2.57	0.26
5	1	89.51	90.81	85.97	92.08
	2	79.63	87.19	88.12	93.15
	Mean	84.57	89.00	87.05	92.62
	Std	6.99	2.56	1.52	0.76
	S.E.	4.94	1.81	1.08	0.53
7	1	86.45	92.75	89.97	91.48
	2	85.67	87.53	90.46	93.46
	Mean	86.06	90.14	90.22	92.47
	Std	0.55	3.69	0.35	1.40
	S.E.	0.39	2.61	0.24	0.99
14	1	87.04	88.51	88.74	89.55
	2	90.78	88.72	83.63	91.42
	Mean	88.91	88.62	86.19	90.49
	Std	2.64	0.15	3.61	1.32
	S.E.	1.87	0.10	2.55	0.94
Overall Mean		86.76	89.00	87.77	92.17
Over all standard dev		3.50	2.81	2.58	1.34
Over all standard error		1.24	0.99	0.91	0.47

	Appendix 2.3/1.9 : ADC of protein using AIA as a marker (experiment 3/1).				
		6 hours fecal collection		24 hours fecal collection	
Days	Replication	Diet with Cr.	Diet with microtracer	Diet with Cr.	Diet with microtracer
3	1	95.85	88.36	96.55	90.60
	2	95.14	95.65	96.98	92.09
	Mean	95.50	92.01	96.77	91.35
	Std	0.50	5.15	0.30	1.05
	S.E.	0.35	3.64	0.22	0.74
5	1	95.11	91.39	96.29	93.41
	2	96.49	94.49	94.90	93.57
	Mean	95.80	92.94	95.60	93.49
	Std	0.98	2.19	0.98	0.11
	S.E.	0.69	1.55	0.70	0.08
7	1	96.80	95.09	94.47	93.66
	2	95.31	90.12	96.61	-
	Mean	96.06	92.61	95.54	93.66
	Std	1.05	3.51	1.51	-
	S.E.	0.74	2.48	1.07	-
14	1	96.42	93.02	96.94	90.68
	2	94.91	94.08	94.33	94.81
	Mean	95.67	93.55	95.64	92.75
	Std	1.07	0.75	1.85	2.92
	S.E.	0.76	0.53	1.31	2.06
Overall Mean		95.75	92.78	95.88	92.69
Over all standard dev		0.74	2.59	1.12	1.61
Over all standard error		0.26	1.06	0.40	0.72

Appendix 2.3/1.10 : ADC (%) of dry matter using microtracer F-Ni as a marker (expt.3/1)					
		6 hours interval		24 hours interval	
Days	Replication	Stripping	Fecal collection	Stripping	Fecal collection
3	1	67.45	48.95	47.21	75.22
	2	57.80	72.22	67.08	74.12
	Mean	62.63	60.59	57.15	74.67
	Std	6.82	16.45	14.05	0.78
	S.E.	4.82	11.64	9.93	0.55
5	1	69.16	68.83	45.77	75.42
	2	39.47	60.54	57.42	74.57
	Mean	54.32	64.69	51.60	75.00
	Std	20.99	5.86	8.24	0.60
	S.E.	14.85	4.15	5.82	0.42
7	1	60.46	75.00	64.53	70.00
	2	55.70	59.51	62.98	77.71
	Mean	58.08	67.26	63.76	73.86
	Std	3.37	10.95	1.10	5.45
	S.E.	2.38	7.74	0.77	3.86
14	1	61.12	61.34	61.19	64.69
	2	72.43	64.74	45.99	66.05
	Mean	66.78	63.04	53.59	65.37
	Std	8.00	2.40	10.75	0.96
	S.E.	5.66	1.70	7.60	0.68
Overall Mean		60.44	63.89	56.52	72.22
Over all standard dev		10.29	8.26	8.89	4.75
Over all standard error		4.20	3.26	3.14	1.94

Appendix 2.3/1.11 : ADC (%) of dry matter using chromic oxide as a marker (expt. 3/1).					
		6 hours interval		24 hours interval	
Days	Replication	Stripping	Fecal collection	Stripping	Fecal collection
3	1	50.19	64.63	62.32	73.90
	2	56.23	65.88	52.64	71.02
	Mean	53.21	65.26	57.48	72.46
	Std	4.27	0.88	6.84	2.04
	S.E.	3.02	0.63	4.84	1.44
5	1	59.88	56.23	60.96	69.16
	2	69.80	70.32	57.93	72.04
	Mean	64.84	63.28	59.45	70.60
	Std	7.01	9.96	2.14	2.04
	S.E.	4.96	7.05	1.52	1.44
7	1	49.61	64.34	57.52	71.30
	2	50.76	64.91	60.61	67.54
	Mean	50.19	64.63	59.07	69.42
	Std	0.81	0.40	2.18	2.66
	S.E.	0.57	0.29	1.54	1.88
14	1	68.49	68.25	58.93	72.66
	2	66.01	67.42	61.18	73.50
	Mean	67.25	67.84	60.06	73.08
	Std	1.75	0.59	1.59	0.59
	S.E.	1.24	0.41	1.13	0.42
Overall Mean		58.87	65.25	59.01	71.39
Over all standard dev		8.43	4.18	3.07	2.16
Over all standard error		2.98	1.48	1.09	0.76

Appendix 2.3/1.12 : ADC (%) of dry matter using AIA as a marker (experiment 3/1)					
		6 hours fecal collection		24 hours fecal collection	
Days	Replication	Diets with Cr.	Diets with microtracer	Diets with Cr.	Diets with microtracer
3	1	86.22	62.19	88.77	64.84
	2	84.54	84.00	90.13	71.31
	Mean	85.38	73.10	89.45	68.08
	Std	1.19	15.42	0.96	4.57
	S.E.	0.84	10.90	0.68	3.24
5	1	81.03	70.79	86.92	79.55
	2	88.27	83.03	84.66	76.09
	Mean	84.65	76.91	85.79	77.82
	Std	5.12	8.65	1.60	2.45
	S.E.	3.62	6.12	1.13	1.73
7	1	89.23	83.09	82.98	77.67
	2	85.12	67.92	88.90	-
	Mean	87.18	75.51	85.94	77.67
	Std	2.91	10.73	4.19	-
	S.E.	2.06	7.58	2.96	-
14	1	89.41	76.51	88.62	68.50
	2	84.87	81.48	79.75	79.49
	Mean	87.14	79.00	84.19	74.00
	Std	3.21	3.51	6.27	7.77
	S.E.	2.27	2.49	4.44	5.50
Overall Mean		86.09	76.13	86.34	73.92
Over all standard dev		2.83	8.25	3.58	5.78
Over all standard error		1.00	2.92	1.27	2.18

Appendix 2.3/2.1 : Chromic oxide recovery (%) from feed and feces sample (Experiment 3/2)							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)
3	1	3.3464	3.3318	4.8910	4.6574	4.0442	3.8836
	2	3.3722	3.9742	4.8618	3.8544	3.7960	3.6792
	Mean	3.3593	3.6530	4.8764	4.2559	3.9201	3.7814
	S.E.	0.01	0.32	0.01	0.40	0.12	0.10
5	1	3.0106	3.5508	4.3946	4.6282	3.8690	3.9274
	2	3.5070	3.8720	4.2778	4.8764	4.0004	4.4530
	Mean	3.2588	3.7114	4.3362	4.7523	3.9347	4.1902
	S.E.	0.25	0.16	0.06	0.12	0.07	0.26
7	1	3.9742	4.1932	4.2632	4.2340	3.7814	3.6208
	2	3.5946	3.7698	4.9348	4.7596	3.6062	3.8836
	Mean	3.7844	3.9815	4.5990	4.4968	3.6938	3.7522
	S.E.	0.19	0.21	0.34	0.26	0.09	0.13
14	1	3.9012	3.8720	3.8982	5.0808	3.7376	3.8544
	2	4.1640	4.2370	3.9128	4.5260	3.1682	3.7668
	Mean	4.0326	4.0545	3.9055	4.8034	3.4529	3.8106
	S.E.	0.13	0.18	0.01	0.28	0.28	0.04
Overall Mean		3.6088	3.8501	4.4293	4.5571	3.7504	3.8836
Overall standard Error		0.14	0.11	0.15	0.14	0.10	0.09
			D-1(with Cr)	D-3(with both)			
Chromic oxide content (%, w/w) in diets			1.63	1.60			

Appendix 2.3/2. 2 : Microtracer F-Ni recovery (%) from feed and feces samples (Experiment 3/2).							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)
3	1	2.2480	2.5950	2.4191	3.1806	2.8051	2.9079
	2	2.9427	2.6323	3.6863	3.8746	2.2459	2.8155
	Mean	2.5954	2.6137	3.0527	3.5276	2.5255	2.8617
	S.E.	0.35	0.02	0.63	0.35	0.28	0.05
5	1	2.3166	3.1023	3.0001	3.8262	2.6810	2.8810
	2	3.1666	2.8576	3.3909	3.7337	2.8554	2.7542
	Mean	2.7416	2.9800	3.1955	3.7800	2.7682	2.8176
	S.E.	0.42	0.12	0.20	0.05	0.09	0.06
7	1	2.2890	2.2592	3.1923	3.3683	2.4482	2.8153
	2	2.2333	3.3694	3.8284	3.1493	2.3195	2.6508
	Mean	2.2612	2.8143	3.5104	3.2588	2.3839	2.7331
	S.E.	0.03	0.56	0.32	0.11	0.06	0.08
14	1	2.0441	2.3135	3.3107	3.1307	3.2389	2.1465
	2	3.0572	2.1448	3.1115	4.1888	1.8394	3.0555
	Mean	2.5507	2.2292	3.2111	3.6598	2.5392	2.6010
	S.E.	0.51	0.08	0.10	0.53	0.70	0.45
Overall Mean		2.5372	2.6593	3.2424	3.5565	2.5542	2.7533
Overall standard Error		0.16	0.15	0.15	0.14	0.15	0.10
Microtracer content (%, w/w) in diets.							
	Replication	D-2(with F-Ni	D-3(with both)				
	1	1.0909	1.1544				
	2	1.4415	1.0925				
	3	1.0555	1.1878				
	4	1.2255	1.1898				
	Mean	1.2034	1.1561				
	S.E.	0.09	0.02				

Appendix 2. 3/2. 3 : Protein content (%) of diets and feces samples (Experiment 3/2).										
		6 hours fecal collection			24 hours fecal collection			24 hours rectum collection		
Days	Replication	T-1(Cr oxide)	T-2(F-Ni)	T-3(Cr + F-Ni)	T-1(Cr oxide)	T-2(F-Ni)	T-3(Cr + F-Ni)	T-1(Cr oxide)	T-2(F-Ni)	T-3(Cr + F-Ni)
3	1	15.94	15.94	15.19	15.94	17.44	14.13	15.94	15.44	16.50
	2	15.44	16.13	16.19	17.00	15.19	13.94	16.38	16.88	17.56
	Mean	15.69	16.04	15.69	16.47	16.32	14.04	16.16	16.16	17.03
	Std	0.35	0.13	0.71	0.75	1.59	0.13	0.31	1.02	0.75
	S.E.	0.25	0.10	0.50	0.53	1.13	0.10	0.22	0.72	0.53
5	1	15.06	15.44	15.44	16.06	17.06	16.13	15.81	17.06	18.81
	2	16.19	16.44	15.63	15.38	17.13	16.88	17.00	17.44	17.50
	Mean	15.63	15.94	15.54	15.72	17.10	16.51	16.41	17.25	18.16
	Std	0.80	0.71	0.13	0.48	0.05	0.53	0.84	0.27	0.93
	S.E.	0.57	0.50	0.10	0.34	0.04	0.38	0.59	0.19	0.65
7	1	16.19	18.50	16.50	15.50	14.13	16.81	18.19	19.56	16.56
	2	15.56	15.75	16.00	15.69	15.19	14.75	17.19	18.06	18.81
	Mean	15.88	17.13	16.25	15.60	14.66	15.78	17.69	18.81	17.69
	Std	0.45	1.94	0.35	0.13	0.75	1.46	0.71	1.06	1.59
	S.E.	0.32	1.38	0.25	0.10	0.53	1.03	0.50	0.75	1.13
14	1	15.50	17.50	16.19	14.88	15.56	15.00	17.25	18.06	17.25
	2	15.06	14.13	17.13	14.31	16.75	15.94	17.94	17.06	18.19
	Mean	15.28	15.82	16.66	14.60	16.16	15.47	17.60	17.56	17.72
	Std	0.31	2.38	0.66	0.40	0.84	0.66	0.49	0.71	0.66
	S.E.	0.22	1.68	0.47	0.28	0.60	0.47	0.34	0.50	0.47

Protein content (%) in diets			
Repli	D-1(Croxi)	D-2(F-Ni)	D-3(Cr+F-Ni)
1	48.06	47.63	46.50
2	47.13	47.50	48.19
3	48.38	49.75	49.00
Mean	47.86	48.29	47.90
Std	0.65	1.26	1.28
S.E.	0.37	0.73	0.74

Appendix 2.3/2.4 : Ash content (%) in diets and feces samples (Experiment 3/2).							
		6 hours fecal collection			24 hours fecal collection		
Days	Replication	T-1(Cr oxide)	T-2(F-Ni)	T-3(Cr + F-Ni)	T-1(Cr oxide)	T-2(F-Ni)	T-3(Cr + F-Ni)
3	1	22.6206	23.6397	30.2759	22.1622	23.8052	29.4445
	2	22.4892	26.2565	29.5216	22.2010	24.7229	27.8789
	Mean	22.5549	24.9481	29.8988	22.1816	24.2641	28.6617
	Std	0.0929	1.8504	0.5334	0.0274	0.6489	1.1070
	S.E.	0.0657	1.3084	0.3772	0.0194	0.4589	0.7828
5	1	22.0737	23.6335	31.2817	21.7338	23.7783	27.4287
	2	22.2922	27.5069	30.0363	21.8063	24.4680	29.7369
	Mean	22.1830	25.5702	30.6590	21.7701	24.1232	28.5828
	Std	0.1545	2.7389	0.8806	0.0513	0.4877	1.6321
	S.E.	0.1092	1.9367	0.6227	0.0362	0.3448	1.1541
7	1	21.5118	21.4496	28.0634	21.3205	23.1416	27.0898
	2	22.0201	25.6815	30.6948	21.1728	23.9970	30.2444
	Mean	21.7660	23.5656	29.3791	21.2467	23.5693	28.6671
	Std	0.3594	2.9924	1.8607	0.1044	0.6049	2.2306
	S.E.	0.2541	2.1160	1.3157	0.0738	0.4277	1.5773
14	1	20.4196	23.0236	30.1795	19.2298	24.7421	29.6000
	2	21.2409	24.4523	27.3873	21.6784	21.3924	28.1537
	Mean	20.8303	23.7380	28.7834	20.4541	23.0673	28.8769
	Std	0.5807	1.0102	1.9744	1.7314	2.3686	1.0227
	S.E.	0.4106	0.7143	1.3961	1.2243	1.6748	0.7231
	Overall Mean	21.8335	24.4555	29.6801	21.4131	23.7559	28.7747
	Overall S.E.	0.26	0.69	0.47	0.37	0.39	0.45
Ash content (%) in diets							
Replication	D-1(Cr oxide)	D-2(F-Ni)	D-3(Cr+F-Ni)				
1	8.9746	10.1586	10.8184				
2	9.4328	10.0882	10.6701				
3	8.9629	10.2078	10.753				
4	9.0073	9.7968	10.7942				
Mean	9.0944	10.0629	10.7589				
Std	0.23	0.18	0.07				
S.E.	0.11	0.09	0.03				

Appendix 2.3/2.5 : AIA content (%) in diets and feces samples (Experiment 3/2).							
		6 hours faecal collection			24 hours faecal collection		
Days	Replication	T-1(Cr oxide)	T-2 (F-Ni)	T-3(Cr + F-Ni)	T-1(Cr oxide)	T-2 (F-Ni)	T-3(Cr + F-Ni)
3	1	1.8128	1.7439	6.2938	2.5445	2.2830	6.4080
	2	2.2311	3.1796	3.2422	2.0979	2.2759	4.4832
	Mean	2.0220	2.4618	4.7680	2.3212	2.2795	5.4456
	Std	0.30	1.02	2.16	0.32	0.01	1.36
	S.E.	0.21	0.72	1.53	0.22	0.00	0.96
5	1	2.2453	2.6861	4.6483	2.0723	3.6766	6.0476
	2	1.9074	2.9045	4.5738	2.6021	3.5950	7.4899
	Mean	2.0764	2.7953	4.6111	2.3372	3.6358	6.7688
	Std	0.24	0.15	0.05	0.37	0.06	1.02
	S.E.	0.17	0.11	0.04	0.26	0.04	0.72
7	1	2.4985	2.1055	3.0065	2.5941	2.4905	6.8484
	2	2.5949	3.9137	4.6590	3.0749	3.6779	5.6551
	Mean	2.5467	3.0096	3.8328	2.8345	3.0842	6.2518
	Std	0.07	1.28	1.17	0.34	0.84	0.84
	S.E.	0.05	0.90	0.83	0.24	0.59	0.60
14	1	2.3605	2.3761	7.2037	2.2188	2.9694	5.0595
	2	2.8746	2.7569	3.6994	4.7133	2.3790	5.3361
	Mean	2.6176	2.5665	5.4516	3.4661	2.6742	5.1978
	Std	0.36	0.27	2.48	1.76	0.42	0.20
	S.E.	0.26	0.19	1.75	1.25	0.30	0.14
Over all Mean		2.3156	2.7083	4.6658	2.7397	2.9184	5.9160
Over all standard error		0.12	0.24	0.51	0.31	0.23	0.35
Acid Insoluble Ash contents (%) in diets							
Replication	D-1(Cr oxide)	D-2(F-Ni)	D-3(Cr + F-Ni)				
1	0.3078	1.2346	1.3369				
2	0.3567	0.8432	1.4082				
3	0.3349	1.2721	1.5681				
4		1.3278	1.2162				
Mean	0.3331	1.1694	1.3824				
Std	0.02	0.22	0.15				
S.E.	0.01	0.11	0.07				

Appendix 2.3/2.6 : ADC (%) of protein using microtracer F-Ni as a marker (Experiment 3/2).							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)
3	1	82.35	85.87	82.04	89.28	86.28	86.30
	2	86.34	85.16	89.73	91.32	81.23	84.95
	Mean	84.35	85.52	85.89	90.30	83.76	85.63
	Std	2.82	0.50	5.44	1.44	3.57	0.95
	S.E.	2.00	0.36	3.85	1.02	2.52	0.68
5	1	83.39	87.99	85.83	89.83	84.14	84.24
	2	87.06	86.80	87.41	89.09	84.78	84.64
	Mean	85.23	87.40	86.62	89.46	84.46	84.44
	Std	2.60	0.84	1.12	0.52	0.45	0.28
	S.E.	1.83	0.60	0.79	0.37	0.32	0.20
7	1	79.86	82.37	88.97	87.95	80.09	85.80
	2	82.43	88.54	90.11	88.70	80.56	82.91
	Mean	81.15	85.46	89.54	88.33	80.33	84.36
	Std	1.82	4.36	0.81	0.53	0.33	2.04
	S.E.	1.28	3.08	0.57	0.38	0.24	1.45
14	1	78.62	83.09	88.29	88.44	86.11	80.60
	2	88.48	80.72	86.59	90.81	76.89	85.63
	Mean	83.55	81.91	87.44	89.63	81.50	83.12
	Std	6.97	1.68	1.20	1.68	6.52	3.56
	S.E.	4.93	1.19	0.85	1.19	4.61	2.51
Overall Mean		83.57	85.07	87.37	89.43	82.51	84.38
Overall Standard dev.		3.49	2.79	2.62	1.16	3.34	1.86
Overall standard error		1.42	1.14	1.07	0.48	1.36	0.76

Appendix 2. 3/2. 7 : ADC (%) of protein using chromic oxide as a marker (Experiment 3/2).							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)
3	1	83.78	84.77	88.90	89.87	86.58	85.81
	2	84.41	86.39	88.09	87.92	85.30	84.06
	Mean	84.10	85.58	88.50	88.90	85.94	84.94
	Std	0.45	1.15	0.57	1.38	0.91	1.24
	S.E.	0.32	0.81	0.41	0.98	0.64	0.88
5	1	82.96	85.48	87.55	88.36	86.08	84.00
	2	84.28	86.52	87.76	88.44	85.53	86.87
	Mean	83.62	86.00	87.66	88.40	85.81	85.44
	Std	0.93	0.74	0.15	0.06	0.39	2.03
	S.E.	0.66	0.52	0.11	0.04	0.27	1.44
7	1	86.13	86.86	87.62	86.74	83.62	84.72
	2	85.26	85.82	89.17	89.65	83.77	83.82
	Mean	85.70	86.34	88.40	88.20	83.70	84.27
	Std	0.62	0.74	1.10	2.06	0.11	0.64
	S.E.	0.43	0.52	0.78	1.45	0.07	0.45
14	1	86.47	86.03	87.00	90.14	84.24	85.05
	2	87.68	86.50	87.54	88.24	80.71	83.87
	Mean	87.08	86.27	87.27	89.19	82.48	84.46
	Std	0.86	0.33	0.38	1.34	2.50	0.83
	S.E.	0.61	0.24	0.27	0.95	1.77	0.59
Overall Mean		85.12	86.05	87.95	88.67	84.48	84.78
Overall Standard dev.		1.56	0.68	0.74	1.15	1.86	1.09
Overall standard error		0.55	0.24	0.26	0.40	0.66	0.39

Appendix 2. 3/2. 8 : ADC (%) of protein using AIA as a marker (Experiment 3/2).							
		6 hour fecal collection			24 hours fecal collection		
Days	Replication	T-1(Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-1(Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)
3	1	93.88	77.87	93.03	95.64	81.50	93.64
	2	95.18	87.72	85.59	94.36	83.84	91.03
	Mean	94.53	82.80	89.31	95.00	82.67	92.34
	Std	0.92	6.97	5.26	0.91	1.65	1.85
	S.E.	0.65	4.93	3.72	0.64	1.17	1.31
5	1	95.33	86.08	90.41	94.61	88.76	92.30
	2	94.09	86.29	90.14	95.89	88.46	93.50
	Mean	94.71	86.19	90.28	95.25	88.61	92.90
	Std	0.88	0.15	0.19	0.91	0.21	0.85
	S.E.	0.62	0.11	0.13	0.64	0.15	0.60
7	1	95.49	78.72	84.16	95.84	86.26	92.92
	2	92.83	90.25	90.09	96.45	90.00	92.47
	Mean	94.16	84.49	87.13	96.15	88.13	92.70
	Std	1.88	8.15	4.19	0.43	2.64	0.32
	S.E.	1.33	5.76	2.97	0.30	1.87	0.22
14	1	95.43	82.16	93.51	95.33	87.31	91.44
	2	96.35	87.59	86.64	97.89	82.95	91.38
	Mean	95.89	84.88	90.08	96.61	85.13	91.41
	Std	0.65	3.84	4.86	1.81	3.08	0.04
	S.E.	0.46	2.72	3.44	1.28	2.18	0.03
Overall Mean		94.82	84.59	89.20	95.75	86.14	92.34
Overall Standard Dev.		1.13	4.50	3.41	1.10	3.06	0.99
Overall Standard error		0.40	1.84	1.39	0.39	1.25	0.40

Appendix 2.3/2.9 : ADC (%) of dry matter using microtracer F-Ni as a marker (Experiment 3/2).							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)
3	1	46.52	55.45	50.26	63.65	57.10	60.24
	2	59.11	56.08	67.36	70.16	46.32	58.94
	Mean	52.82	55.77	58.81	66.91	51.71	59.59
	Std	8.90	0.45	12.09	4.60	7.62	0.92
	S.E.	6.29	0.32	8.55	3.26	5.39	0.65
5	1	48.06	62.73	59.89	69.78	55.12	59.87
	2	62.00	59.54	64.51	69.04	57.86	57.96
	Mean	55.03	61.14	62.20	69.41	56.49	58.92
	Std	9.86	2.26	3.27	0.52	1.94	1.35
	S.E.	6.97	1.60	2.31	0.37	1.37	0.96
7	1	47.43	48.43	62.31	65.68	50.85	58.94
	2	46.12	65.69	68.57	63.29	48.03	56.48
	Mean	46.78	57.06	65.44	64.49	49.44	57.71
	Std	0.93	12.20	4.43	1.69	1.99	1.74
	S.E.	0.65	8.63	3.13	1.20	1.41	1.23
14	1	41.01	49.96	63.65	63.07	62.85	46.14
	2	60.64	46.10	61.33	72.39	34.58	62.16
	Mean	50.83	48.03	62.49	67.73	48.72	54.15
	Std	13.88	2.73	1.64	6.59	19.99	11.33
	S.E.	9.81	1.93	1.16	4.66	14.13	8.01
Overall Mean		51.36	55.55	62.24	67.13	51.59	57.59
Overall standard Dev.		7.96	6.93	5.65	3.64	8.78	4.92
Overall standard Error		3.25	2.83	2.31	1.49	3.58	2.01

Appendix 2.3/2.10 : ADC (%) of dry matter using chromic oxide as a marker (Experiment 3/2).							
		6 hour fecal collection		24 hours fecal collection		24 hours rectum collection	
Days	Replication	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)	T-1 (Cr)	T-3(F-Ni+Cr)
3	1	51.29	51.98	66.67	65.65	59.70	58.80
	2	51.66	59.74	66.47	58.49	57.06	56.51
	Mean	51.48	55.86	66.57	62.07	58.38	57.66
	Std	0.26	5.49	0.14	5.06	1.87	1.62
	S.E.	0.19	3.88	0.10	3.58	1.32	1.15
5	1	45.86	54.94	62.91	65.43	57.87	59.26
	2	53.52	58.68	61.90	67.19	59.25	64.07
	Mean	49.69	56.81	62.41	66.31	58.56	61.67
	Std	5.42	2.64	0.71	1.24	0.98	3.40
	S.E.	3.83	1.87	0.50	0.88	0.69	2.41
7	1	58.99	61.84	61.77	62.21	56.89	55.81
	2	54.65	57.56	66.97	66.38	54.80	58.80
	Mean	56.82	59.70	64.37	64.30	55.85	57.31
	Std	3.07	3.03	3.68	2.95	1.48	2.11
	S.E.	2.17	2.14	2.60	2.08	1.05	1.50
14	1	58.22	58.68	58.19	68.51	56.39	58.49
	2	60.85	62.24	58.34	64.65	48.55	57.52
	Mean	59.54	60.46	58.27	66.58	52.47	58.01
	Std	1.86	2.52	0.11	2.73	5.54	0.69
	S.E.	1.32	1.78	0.08	1.93	3.92	0.49
Overall Mean		54.38	58.21	62.90	64.81	56.31	58.66
Overall standard Dev.		4.90	3.43	3.56	3.15	3.50	2.50
Overall standard Error		1.73	1.21	1.26	1.12	1.24	0.88

Appendix 2.3/2.11 : ADC (%) of dry matter using AIA as a marker (Experiment 3/2).							
		6 hour fecal collection			24 hours fecal collection		
Days	Replication	T-1 (Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)	T-1 (Cr)	T-2 (F-Ni)	T-3(F-Ni+Cr)
3	1	81.63	32.94	78.04	86.91	48.78	78.43
	2	85.07	63.22	57.36	84.12	48.62	69.16
	Mean	83.35	48.08	67.70	85.52	48.70	73.80
	Std	2.43	21.41	14.62	1.97	0.11	6.55
	S.E.	1.72	15.14	10.34	1.40	0.08	4.64
5	1	85.16	56.46	70.26	83.93	68.19	77.14
	2	82.56	59.74	69.78	87.20	67.47	81.54
	Mean	83.86	58.10	70.02	85.57	67.83	79.34
	Std	1.84	2.32	0.34	2.31	0.51	3.11
	S.E.	1.30	1.64	0.24	1.63	0.36	2.20
7	1	86.67	44.46	54.02	87.16	53.05	79.81
	2	87.16	70.12	70.33	89.17	68.20	75.55
	Mean	86.92	57.29	62.18	88.17	60.63	77.68
	Std	0.35	18.14	11.53	1.42	10.71	3.01
	S.E.	0.24	12.83	8.15	1.00	7.57	2.13
14	1	85.89	50.78	80.81	84.99	60.62	72.68
	2	88.41	57.58	62.63	92.93	50.84	74.09
	Mean	87.15	54.18	71.72	88.96	55.73	73.39
	Std	1.78	4.81	12.86	5.61	6.92	1.00
	S.E.	1.26	3.40	9.09	3.97	4.89	0.70
Overall Mean		85.32	54.41	67.90	87.05	58.22	76.05
Overall standard Dev.		2.28	11.59	9.38	2.97	8.89	4.04
Overall standard Error		0.81	4.73	3.83	1.05	3.63	1.65

Appendix Three

(Experiment Four)

Appendix 3.4.1 : Chromic oxide content (% w/w) in feed and feces samples (4th expt.)					
		Feces related with F.M.		Feces related with S. M.	
Day	Replicate	Reference Diet	Test Diet	Reference Diet	Test Diet
3	1	5.6502	8.1439	6.0444	5.0662
	2	5.9714	8.0840	6.5992	4.5844
	Mean	5.8108	8.1140	6.3218	4.8253
	Std	0.23	0.04	0.39	0.34
	S.E.	0.16	0.03	0.28	0.24
5	1	6.7189	8.4768	5.0078	5.2852
	2	6.1758	8.7191	5.0954	4.6866
	Mean	6.4474	8.5980	5.0516	4.9859
	Std	0.38	0.17	0.06	0.42
	S.E.	0.27	0.12	0.04	0.30
7	1	5.8400	7.0525	6.1758	5.0224
	2	5.9276	8.4951	6.7014	5.1684
	Mean	5.8838	7.7738	6.4386	5.0954
	Std	0.06	1.02	0.37	0.10
	S.E.	0.04	0.72	0.26	0.07
10	1	5.7086	7.6796	5.4312	6.0152
	2	5.9568	6.9788	6.3072	4.0150
	Mean	5.8327	7.3292	5.8692	5.0151
	Std	0.18	0.50	0.62	1.41
	S.E.	0.12	0.35	0.44	1.00
Overall Mean		5.9937	7.9537	5.9203	4.9804
Over all Std		0.34	0.66	0.66	0.58
Over all S.E.		0.12	0.23	0.23	0.21
		Diets related with F.M.		Diets related with S. M.	
		Reference Diet	Test Diet	Reference Diet	Test Diet
Cromic oxide cont. in diet		1.0512	1.2848	1.2264	1.2994

Appendix 3.4.2 : Microtracer content (% w/w) in feed and feces samples (4th ex.)					
		Feces related with F.M.		Feces related with S. M.	
Day	Replicate	Reference Diet	Test Diet	Reference Diet	Test Diet
3	1	8.4238	5.2447	5.2276	5.4093
	2	7.1417	7.4184	5.3535	3.9227
	Mean	7.7828	6.3316	5.2906	4.6660
	Std	0.91	1.54	0.09	1.05
	S.E.	0.64	1.09	0.06	0.74
5	1	7.3385	5.3786	6.8456	3.9790
	2	5.5822	5.8492	7.6255	4.6193
	Mean	6.4604	5.6139	7.2356	4.2992
	Std	1.24	0.33	0.55	0.45
	S.E.	0.88	0.24	0.39	0.32
7	1	7.9731	5.7337	6.4084	5.5070
	2	7.3842	4.8917	7.3924	6.3515
	Mean	7.6787	5.3127	6.9004	5.9293
	Std	0.42	0.60	0.70	0.60
	S.E.	0.29	0.42	0.49	0.42
10	1	5.0048	8.6916	6.4566	6.7815
	2	5.2985	6.5661	4.7459	4.7740
	Mean	5.1517	7.6289	5.6013	5.7778
	Std	0.21	1.50	1.21	1.42
	S.E.	0.15	1.06	0.86	1.00
Overall Mean		6.7684	6.2218	6.2569	5.1680
Over all Std		1.29	1.28	1.05	1.04
Over all S.E.		0.46	0.45	0.37	0.37
		Diets related with F.M.		Diets related with S. M.	
		Reference Diet	Test Diet	Reference Diet	Test Diet
Microtracer content in diets		1.1383	1.1567	0.9092	0.9859

Appendix 3.4.3 : Protein (N x 6.25) content (%) in feed and feces samples (4th ex.).					
		Feces related with F.M.		Feces related with S. M.	
Day	Replicate	Reference Diet	Test Diet	Reference Diet	Test Diet
3	1	12.50	17.69	12.88	8.44
	2	10.75	17.69	10.75	7.94
	Mean	11.63	17.69	11.82	8.19
	Std	1.24	0.00	1.51	0.35
	S.E.	0.88	0.00	1.06	0.25
5	1	12.44	15.75	12.56	10.81
	2	11.88	17.06	12.94	8.31
	Mean	12.16	16.41	12.75	9.56
	Std	0.40	0.93	0.27	1.77
	S.E.	0.28	0.66	0.19	1.25
7	1	11.81	13.25	15.06	9.06
	2	15.88	20.88	13.63	8.38
	Mean	13.85	17.07	14.35	8.72
	Std	2.88	5.40	1.01	0.48
	S.E.	2.04	3.81	0.71	0.34
10	1	12.75	17.38	14.06	8.81
	2	10.94	17.44	13.19	9.38
	Mean	11.85	17.41	13.63	9.10
	Std	1.28	0.04	0.62	0.40
	S.E.	0.91	0.03	0.44	0.28
Overall Mean		12.37	17.14	13.13	8.89
Over all Std		1.59	2.13	1.25	0.90
Over all S.E.		0.56	0.75	0.44	0.32
		Diets related with F.M.		Diets related with S. M.	
		Reference Diet	Test Diet	Reference Diet	Test Diet
Protein content in diets		60.00	63.94	55.31	50.63

Appendix 3.4.4 : Ash content (%) in diets and feces samples (4th experiment).					
		Feces related with F.M.		Feces related with S. M.	
Day	Replicate	Reference Diet	Test Diet	Reference Diet	Test Diet
3	1	30.1588	43.5659	29.1341	22.3830
	2	32.1291	43.3563	38.4215	21.1237
	Mean	31.1440	43.4611	33.7778	21.7534
	Std	1.3932	0.1482	6.5672	0.8905
	S.E.	0.9852	0.1048	4.6437	0.6297
5	1	27.2167	44.4658	32.9993	19.4034
	2	32.9578	41.9622	33.4485	20.2901
	Mean	30.0873	43.2140	33.2239	19.8468
	Std	4.0596	1.7703	0.3176	0.6270
	S.E.	2.8706	1.2518	0.2246	0.4434
7	1	27.4685	41.6011	33.1872	21.5574
	2	30.1869	41.7128	35.2582	20.6348
	Mean	28.8277	41.6570	34.2227	21.0961
	Std	1.9222	0.0790	1.4644	0.6524
	S.E.	1.3592	0.0558	1.0355	0.4613
10	1	26.2025	44.9190	33.0342	22.6856
	2	28.7355	42.5730	35.7937	24.6109
	Mean	27.4690	43.7460	34.4140	23.6483
	Std	1.7911	1.6589	1.9513	1.3614
	S.E.	1.2665	1.1730	1.3797	0.9627
Overall Mean		29.3820	43.0195	33.9096	21.5861
Over all Std		2.40	1.26	2.70	1.63
Over all S.E.		0.85	0.45	0.95	0.58
		Diets related with F.M.		Diets related with S. M.	
		Reference Diet	Test Diet	Reference Diet	Test Diet
Ash content in diets		9.0315	11.6826	9.3646	9.7639

Appendix 3.4.5 : AIA content (%) in diet and feces samples (4th experiment)					
		Feces related with F.M.		Feces related with S. M.	
Day	Replicate	Reference Diet	Test Diet	Reference Diet	Test Diet
3	1	7.0095	11.9936	5.7978	6.6405
	2	9.5553	8.3645	10.7162	6.5012
	Mean	8.2824	10.1791	8.2570	6.5709
	Std	1.80	2.57	3.48	0.10
	S.E.	1.27	1.81	2.46	0.07
5	1	6.0980	8.9984	7.7396	5.7288
	2	8.9593	8.8463	8.1429	6.5735
	Mean	7.5287	8.9224	7.9413	6.1512
	Std	2.02	0.11	0.29	0.60
	S.E.	1.43	0.08	0.20	0.42
7	1	6.3384	7.7323	6.2751	4.8475
	2	7.4390	9.4857	7.2917	4.7866
	Mean	6.8887	8.6090	6.7834	4.8171
	Std	0.78	1.24	0.72	0.04
	S.E.	0.55	0.88	0.51	0.03
10	1	6.2856	11.9197	7.0307	4.8187
	2	8.2995	8.7425	7.2543	5.6977
	Mean	7.2926	10.3311	7.1425	5.2582
	Std	1.42	2.25	0.16	0.62
	S.E.	1.01	1.59	0.11	0.44
Overall Mean		7.4981	9.5104	7.5310	5.6993
Over all Std		1.31	1.59	1.49	0.81
Over all S.E.		0.46	0.56	0.53	0.29
		Diets related with F.M.		Diets related with S. M.	
		Reference Diet	Test Diet	Reference Diet	Test Diet
AIA content in diets		1.6018	1.6855	1.5677	1.7061

Appendix 3.4.6 : ADC (%) of protein in ref., test diets and fish meal ingredient using chromic oxide marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in FM
3	1	96.12	95.63	95.49
	2	96.85	95.60	92.68
	Mean	96.49	95.62	94.09
	Std	0.52	0.02	1.99
	S.E.	0.37	0.01	1.40
5	1	96.76	96.27	95.13
	2	96.63	96.07	94.76
	Mean	96.70	96.17	94.95
	Std	0.09	0.14	0.26
	S.E.	0.06	0.10	0.18
7	1	96.46	96.22	95.66
	2	95.31	95.06	94.48
	Mean	95.89	95.64	95.07
	Std	0.81	0.82	0.83
	S.E.	0.57	0.58	0.59
10	1	96.09	95.45	93.96
	2	96.78	94.98	90.78
	Mean	96.44	95.22	92.37
	Std	0.49	0.33	2.25
	S.E.	0.34	0.24	1.59
Overall Mean		96.38	95.66	94.12
Over all Std		0.52	0.50	1.65
Over all S.E.		0.18	0.18	0.58

Appendix 3.4.7 : ADC (%) of protein in ref., test diets and fish meal ingredient using microtracer F-Ni as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in FM
3	1	97.18	93.90	86.25
	2	97.14	95.69	92.31
	Mean	97.16	94.80	89.28
	Std	0.03	1.27	4.29
	S.E.	0.02	0.90	3.03
5	1	96.78	94.70	89.85
	2	95.96	94.72	91.83
	Mean	96.37	94.71	90.84
	Std	0.58	0.01	1.40
	S.E.	0.41	0.01	0.99
7	1	97.19	95.82	92.62
	2	95.92	92.28	83.79
	Mean	96.56	94.05	88.21
	Std	0.90	2.50	6.24
	S.E.	0.63	1.77	4.41
10	1	95.17	96.38	99.20
	2	96.08	95.20	93.15
	Mean	95.63	95.79	96.18
	Std	0.64	0.83	4.28
	S.E.	0.46	0.59	3.02
Overall Mean		96.43	94.84	91.13
Over all Std		0.75	1.29	4.67
Over all S.E.		0.27	0.46	1.65

Appendix 3.4.8 : ADC (%) of protein in ref., test diets and fish meal ingredient using AIA as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in FM
3	1	95.24	96.11	98.14
	2	97.00	94.43	88.43
	Mean	96.12	95.27	93.29
	Std	1.24	1.19	6.87
	S.E.	0.88	0.84	4.85
5	1	94.55	95.39	97.35
	2	96.46	94.92	91.33
	Mean	95.51	95.16	94.34
	Std	1.35	0.33	4.26
	S.E.	0.95	0.24	3.01
7	1	95.03	95.48	96.53
	2	94.30	94.20	93.97
	Mean	94.67	94.84	95.25
	Std	0.52	0.91	1.81
	S.E.	0.36	0.64	1.28
10	1	94.58	96.16	99.86
	2	96.48	94.74	90.68
	Mean	95.53	95.45	95.27
	Std	1.34	1.00	6.49
	S.E.	0.95	0.71	4.59
Overall Mean		95.46	95.18	94.54
Over all Std		1.04	0.73	4.07
Over all S.E.		0.37	0.26	1.44

Appendix 3.4.9 : ADC (%) of dry matter in ref., test diets and fish meal ingredient using chromic oxide marker (4th exp).				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in FM
3	1	81.39	84.22	90.82
	2	82.40	84.11	88.10
	Mean	81.90	84.17	89.46
	Std	0.71	0.08	1.92
	S.E.	0.50	0.06	1.36
5	1	84.35	84.84	85.98
	2	82.98	85.26	90.58
	Mean	83.67	85.05	88.28
	Std	0.97	0.30	3.25
	S.E.	0.68	0.21	2.30
7	1	82.00	81.78	81.27
	2	82.27	84.88	90.97
	Mean	82.14	83.33	86.12
	Std	0.19	2.19	6.86
	S.E.	0.13	1.55	4.85
10	1	81.59	83.27	87.19
	2	82.35	81.59	79.82
	Mean	81.97	82.43	83.51
	Std	0.54	1.19	5.21
	S.E.	0.38	0.84	3.69
Overall Mean		82.42	83.74	86.84
Over all Std		0.93	1.41	4.30
Over all S.E.		0.33	0.50	1.52

Appendix 3.4.10 : ADC (%) of dry matter in ref., test diets and fish meal ingredient using microtracer F-Ni as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in FM
3	1	86.49	77.95	58.02
	2	84.06	84.41	83.97
	Mean	85.28	81.18	71.00
	Std	1.72	4.57	18.35
	S.E.	1.21	3.23	12.97
5	1	84.49	78.49	64.49
	2	79.61	80.22	81.64
	Mean	82.05	79.36	73.07
	Std	3.45	1.22	12.13
	S.E.	2.44	0.87	8.58
7	1	85.72	79.83	66.09
	2	84.58	76.35	57.15
	Mean	85.15	78.09	61.62
	Std	0.81	2.46	6.32
	S.E.	0.57	1.74	4.47
10	1	77.26	86.69	108.69
	2	78.52	82.38	91.39
	Mean	77.89	84.54	100.04
	Std	0.89	3.05	12.23
	S.E.	0.63	2.16	8.65
Overall Mean		82.59	80.79	76.43
Over all Std		3.56	3.48	18.16
Over all S.E.		1.26	1.23	6.42

Appendix 3.4.11: ADC (%) of dry matter in ref, test diets and fishmeal ingredient by using AIA as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in FM
3	1	77.15	85.98	106.58
	2	83.24	79.90	72.11
	Mean	80.20	82.94	89.35
	Std	4.31	4.30	24.37
	S.E.	3.04	3.04	17.23
5	1	73.73	81.32	99.03
	2	82.12	80.99	78.35
	Mean	77.93	81.16	88.69
	Std	5.93	0.23	14.62
	S.E.	4.20	0.17	10.34
7	1	74.73	78.26	87.20
	2	78.47	82.28	91.17
	Mean	76.60	80.27	89.19
	Std	2.64	2.84	2.81
	S.E.	1.87	2.01	1.98
10	1	74.52	85.89	112.42
	2	80.70	80.77	80.93
	Mean	77.61	83.33	96.68
	Std	4.37	3.62	22.27
	S.E.	3.09	2.56	15.74
Overall Mean		78.08	81.92	90.97
Over all Std		3.66	2.74	14.14
Over all S.E.		1.29	0.97	4.99

Appendix 3.4.12 : ADC (%) of protein in ref., test diets and sunflower meal ingredient using chromic oxide marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in SM
3	1	95.28	95.72	96.75
	2	96.39	95.55	93.59
	Mean	95.84	95.64	95.17
	Std	0.78	0.12	2.23
	S.E.	0.56	0.09	1.58
5	1	94.44	94.75	95.47
	2	94.37	95.45	97.97
	Mean	94.41	95.10	96.72
	Std	0.05	0.49	1.77
	S.E.	0.04	0.35	1.25
7	1	94.59	95.37	97.19
	2	95.49	95.84	96.66
	Mean	95.04	95.61	96.93
	Std	0.64	0.33	0.37
	S.E.	0.45	0.24	0.26
10	1	94.26	96.24	100.86
	2	95.36	94.00	90.83
	Mean	94.81	95.12	95.85
	Std	0.78	1.58	7.09
	S.E.	0.55	1.12	5.02
Overall Mean		95.02	95.37	96.17
Over all Std		0.74	0.70	2.99
Over all S.E.		0.26	0.25	1.06

Appendix 3.4.13 : ADC (%) of protein in ref., test diets and sunflower meal ingredient by using microtracer F-Ni as marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in SM
3	1	95.95	96.96	99.32
	2	96.70	96.06	94.57
	Mean	96.33	96.51	96.95
	Std	0.53	0.64	3.36
	S.E.	0.37	0.45	2.38
5	1	96.98	94.71	89.41
	2	97.21	96.50	94.84
	Mean	97.10	95.61	92.13
	Std	0.16	1.27	3.84
	S.E.	0.12	0.90	2.72
7	1	96.14	96.80	98.34
	2	96.97	97.43	98.50
	Mean	96.56	97.12	98.42
	Std	0.59	0.45	0.11
	S.E.	0.41	0.31	0.08
10	1	96.42	97.43	99.92
	2	95.43	96.17	97.70
	Mean	95.93	96.80	98.81
	Std	0.70	0.89	1.57
	S.E.	0.49	0.63	1.11
Overall Mean		96.48	96.45	96.60
Over all Std		0.61	0.95	3.50
Over all S.E.		0.21	0.33	1.24

Appendix 3.4.14 : ADC of protein in ref., test diets and sunflower meal ingredient by using AIA as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) Protein in SM
3	1	93.70	95.72	100.43
	2	97.16	95.88	92.89
	Mean	95.43	95.80	96.66
	Std	2.45	0.11	5.33
	S.E.	1.73	0.08	3.77
5	1	95.40	93.64	89.53
	2	95.50	95.74	96.30
	Mean	95.45	94.69	92.92
	Std	0.07	1.48	4.79
	S.E.	0.05	1.05	3.38
7	1	93.20	93.70	94.87
	2	94.70	94.10	92.70
	Mean	93.95	93.90	93.79
	Std	1.06	0.28	1.53
	S.E.	0.75	0.20	1.08
10	1	94.33	93.84	92.70
	2	94.85	94.45	93.52
	Mean	94.59	94.15	93.11
	Std	0.37	0.43	0.58
	S.E.	0.26	0.30	0.41
Overall Mean		94.86	94.63	94.12
Over all Std		1.22	0.98	3.21
Over all S.E.		0.43	0.35	1.13

Appendix 3.4.15 : ADC (%) of dry matter in ref., test diets and sunflower meal ingredient using chromic oxide marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in SM
3	1	79.71	74.35	61.84
	2	81.42	71.66	48.89
	Mean	80.57	73.01	55.37
	Std	1.21	1.90	9.16
	S.E.	0.86	1.34	6.47
5	1	75.51	75.41	75.18
	2	75.93	72.27	63.73
	Mean	75.72	73.84	69.46
	Std	0.30	2.22	8.10
	S.E.	0.21	1.57	5.72
7	1	80.14	74.13	60.11
	2	81.70	74.86	58.90
	Mean	80.92	74.50	59.51
	Std	1.10	0.52	0.86
	S.E.	0.78	0.36	0.61
10	1	77.42	78.40	80.69
	2	80.56	67.64	37.49
	Mean	78.99	73.02	59.09
	Std	2.22	7.61	30.55
	S.E.	1.57	5.38	21.60
Overall Mean		79.05	73.59	60.85
Over all Std		2.43	3.16	13.63
Over all S.E.		0.86	1.12	4.82

Appendix 3.4.16 : ADC (%) of dry matter in ref., test diets and sunflower meal ingredient by using microtracer F-Ni as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in SM
3	1	82.61	81.77	79.81
	2	83.02	74.87	55.85
	Mean	82.82	78.32	67.83
	Std	0.29	4.88	16.94
	S.E.	0.21	3.45	11.98
5	1	86.72	75.22	48.39
	2	88.08	78.66	56.68
	Mean	87.40	76.94	52.54
	Std	0.96	2.43	5.86
	S.E.	0.68	1.72	4.14
7	1	85.81	82.10	73.44
	2	87.70	84.48	76.97
	Mean	86.76	83.29	75.21
	Std	1.34	1.68	2.50
	S.E.	0.95	1.19	1.77
10	1	85.92	85.46	84.39
	2	80.84	79.35	75.87
	Mean	83.38	82.41	80.13
	Std	3.59	4.32	6.02
	S.E.	2.54	3.05	4.26
Overall Mean		85.09	80.24	68.93
Over all Std		2.62	3.93	13.27
Over all S.E.		0.93	1.39	4.69

Appendix 3.4.17 : ADC (%) of dry matter in ref., test diets and sunflower meal ingredient by using AIA as a marker.				
Day	Replicate	ADC(%) RD	ADC(%) TD	ADC(%) DM in SM
3	1	72.96	74.31	77.46
	2	85.37	73.76	46.67
	Mean	79.17	74.04	62.07
	Std	8.78	0.39	21.77
	S.E.	6.20	0.28	15.40
5	1	79.74	70.22	48.01
	2	80.75	74.05	58.42
	Mean	80.25	72.14	53.22
	Std	0.71	2.71	7.36
	S.E.	0.50	1.92	5.20
7	1	75.02	64.80	40.95
	2	78.50	64.36	31.37
	Mean	76.76	64.58	36.16
	Std	2.46	0.31	6.77
	S.E.	1.74	0.22	4.79
10	1	77.70	64.59	34.00
	2	78.39	70.06	50.62
	Mean	78.05	67.33	42.31
	Std	0.49	3.87	11.75
	S.E.	0.35	2.73	8.31
Overall Mean		78.55	69.52	48.44
Over all Std		3.73	4.40	14.67
Over all S.E.		1.32	1.56	5.19